QUALITATIVE & SEMI QUANTITATIVE CULTURE ASSAYS OF BACTERIAL BIOBURDEN IN CHRONIC NON HEALING SUPERFICIAL WOUNDS BEFORE AND AFTER ELECTRICAL STIMULATION AND INVITRO STUDY ON THE ANTIBACTERIAL EFFECTS OF ELECTRICAL STIMULATION

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DECLARATION

I, **Dr.S.MAHESH PRABHU,** solemnly declare that this dissertation **"QUALITATIVE & SEMI QUANTITATIVE CULTURE ASSAYS OF BACTERIAL BIOBURDEN IN CHRONIC NON HEALING SUPERFICIAL WOUNDS BEFORE AND AFTER ELECTRICAL STIMULATION AND INVITRO STUDY ON THE ANTIBACTERIAL EFFECTS OF ELECTRICAL STIMULATION"** is the bonafide work done by me at the Department of Microbiology, Government Stanley Medical College and Hospital, Chennai, under the guidance and supervision of **Prof.Dr.P.R.THENMOZHI VALLI, M.D.,** Professor and Head of the Department of Microbiology, Government Stanley Medical College, Chennai - 600 001.

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CERTIFICATE

This is to certify that this dissertation entitled **"QUALITATIVE & SEMI QUANTITATIVE CULTURE ASSAYS OF BACTERIAL BIOBURDEN IN CHRONIC NON HEALING SUPERFICIAL WOUNDS BEFORE AND AFTER ELECTRICAL STIMULATION AND INVITRO STUDY ON THE ANTIBACTERIAL EFFECTS OF ELECTRICAL STIMULATION"** is the bonafide work done by **Dr.S.MAHESH PRABHU** in the Department of Microbiology, Govt. Stanley Medical College & Hospital, Chennai, in partial fulfillment of the regulation for **M.D. (Branch - IV) Microbiology** examination of the Tamil Nadu Dr.M.G.R.Medical University, Chennai, to be held in March 2010.

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INTRODUCTION

Humans are not germ free. Therefore, health is not absence of bacteria but is kindred to an elegant balance among humans, their resident or transient flora and flora of the environment. The intact skin and the mucous membrane are the most eloquent defenses for humans. Any damage or injury to integument disturbs this bastion and its equilibrium with the bacterial flora.

A wound is a breach in the skin and the exposure of subcutaneous tissue following loss of skin integrity provides a moist, warm and nutritive environment that is conducive to microbial colonization and proliferation. Infection occurs when the bacteria accomplish penetration of the subcutaneous tissue and achieve an acute number.

Wounds can be broadly categorized as having either an acute or chronic etiology. Irrespective of the nature of the cutaneous injury, acute wounds are expected to heal within a predictable time frame.

Chronic wounds are those that do not heal with in about a month time with standard treatment (e.g., optimization of nutrition, moist dressings, debridement, infection resolution, repositioning, and off-loading of pressure).

Chronic wounds result from the disruption of the normal healing process and appear to be struck in the inflammatory phase with accumulation of excessive extra cellular matrix components and matrix proteinases. Each wound needs to be evaluated independently to assess which factors may be at play in disturbing the healing process.

Local factors contributing to poor wound healing include infection, tissue hypoxia, repeated trauma, history of irradiation or the presence of

necrotic tissue. Systemic factors include diabetes, ageing, malnutrition, obesity, smoking, immunodeficiency and certain medications.

Although wounds cease to heal for many reasons, perhaps the most common emanates from the effects of wound bioburden[44, 34, 37] due to the sheer quantity of colonizing microbes, the invasive infection, polymicrobial nature and their synergism or the effect of their toxins.

Recently, the terms local infection and critical colonization have been introduced to describe a situation in which the wound has an increasing bacterial burden, which is intermediate between the category of colonization and infection. Wounds that are locally infected or critically colonized will not heal but may not display classic signs of infection. [79, 91] Experimental studies have demonstrated that regardless of the type of microorganism, impairment of wound repair may occur when there are more than $1x10^5$ organisms per gram of tissue.[79, 91,3,34,44,87] The reasons for this lie in the immunological response to chronic wound infection.

The continual presence of bacterial bioburden stimulates the host immune defenses leading to the chronic production of inflammatory mediators, such as prostaglandin E2 and thromboxane. Neutrophils continue to migrate into the wound, releasing cytotoxic enzymes and free oxygen radicals. Thrombosis and vasoconstrictive metabolites cause wound hypoxia, leading to enhanced bacterial proliferation and continued tissue damage. With prolonged bacterial presence in chronic wounds, the bacteria change their pattern of behavior and alter their phenotype and their immune expression. All these factors help them to evade detection by the body's immune system, thus making it difficult to be negated by the host defenses. This development of

"immune tolerance" can create the spurious impression of no infection and may prevent the eradication of microorganisms from the wound clinically.

The number of patients developing chronic non healing wounds is increasing with the world wide increase in lifestyle diseases such as obesity, diabetes and cardiovascular diseases and ageing population. The World Health Organization estimates that by 2025, the number of people with diabetes will reach 300 million. [93] Despite improved treatment, a significant number of diabetic foot ulcers do not heal and eventually lead to amputation. WHO report of Ageing statistics reveals that the number of people aged over 60 years is expected to reach two billion by 2050- the vast majority of these older people will live in the developing world. All these data's show that the prevalence of chronic wounds is likely to increase significantly in the future. The socioeconomic consequences of chronic non healing wound include patient suffering, loss of employment and reduced quality of life. In depth knowledge of chronic wounds is urgently required and would significantly improve treatment and prognosis.

The indiscriminate use of antibiotics, either systemic or topical, for all open wounds would raise health care costs and contribute to the development or selection of multi drug resistant microorganisms; therefore, systemic antibiotics are not an option for prophylactic use in all open wounds and are reserved for proven cases of wound infection. However, proof of infection is fundamentally restricted to acknowledging clinical signs and symptoms in collaboration with qualitative microbiology.

The use of qualitative microbiology alone is flawed because the wounds that become indolent due to bioburden (i.e., critical colonization) but that do not exhibit classically considered signs of infection may go untreated, if quantitative assay is not included along with qualitative assay.

Preparation of wound bed is an important foundation for successful wound treatment by which chronic wounds are made suitable for surgical intervention and get benefited from available wound care technology. The importance of the concept of critical colonization in the science of wound bed preparation is to encourage clinicians to pay closer attention to delayed healing and its assessment. In instances in which healing does not take place, despite optimum treatment, critical colonization should be considered. [87, 91]

As the development of bacterial resistance to antibiotics continues and controversy regarding the use of topical antiseptics persists, the need for identification and development of new antimicrobial agents that are safe and broadly effective and have a lower propensity to induce resistance becomes increasingly critical. [12]

At this juncture, a number of alternative antimicrobial therapies[12] and mechanical adjuncts in wound healing like hyperbaric oxygen therapy, negative pressure therapy, ultrasound and electrical stimulation are included in the management of non healing wounds.[36] Among them electrical stimulation[36,29,43,47,65] may be one of the up coming therapies which is defined as the application of electric current from electrodes placed directly within a wound over sterile moist gauze pad or on skin in close proximity to it. There is evidence from in vitro and in vivo studies[43, 47, and 65,20,23,80] suggesting that electrical stimulation have bacteriostatic and bactericidal effects on microorganisms known to colonize and infect dermal wounds.

In this study, qualitative and semi quantitative aerobic bacterial cultural assay of tissue biopsies from chronic non healing superficial wound patients before and after electrical stimulation is compared with control. In vitro demonstration of the antibacterial effects of electrical stimulation also included in this study.

AIMS AND OBJECTIVES

- To isolate and characterize the bacterial agents causing infections in chronic non healing superficial wounds.
- Estimation of bacterial bio burden in the chronic non healing superficial wounds by semi quantitative culture assay before and after electrical stimulation.
- To compare the effects of electrical stimulation in controlling the bacterial bio burden of chronic wounds with controls
- To compare the healing rates of chronic wounds treated with and without electrical stimulation.
- Clinico-Microbiological correlation of the effects of electrical stimulation on chronic non healing superficial wounds.
- In vitro demonstration of antibacterial effects of electrical stimulation.

REVIEW OF LITERATURE

Background

The Egyptians were performing incision and drainage procedures as early as 2000 BC.The Edwin Smith (1600 BC) and Ebers papyri (1560 BC) are two of the ancient Egypt's most contributions to the management of wounds. Ebers papyri describe the use of fermented goat dung, frogs warmed in oil and therapies for burn wounds and animal bites. The use of frog skin may represent the first described use of a biological dressing in the management of open wounds. Hippocrates (460-377 BC) first described healing by primary and secondary intention and defined the signs of suppuration. Galen (130-200 AD) the most prolific Greek medical writer proposed theory of laudable pus, which stated that the drainage of pus is essential for wound healing.

The earliest discovery of a pathogenic microorganism was probably made by Augustino Bassi (1835). Louis Pasteur (1822-1895) established that fermentation was the result of microbial activity. Joseph Lister (1867) postulated that microbes were responsible for life threatening wound infections and he introduced antiseptic techniques in surgery. Robert Koch (1843-1910) defined the criteria used to attribute a disease to an organism through his famous Koch's postulates. The concept of a 'magic bullet' (Zauber Kugel) that could kill microbes but not their host first became a reality with the discovery of sulphonamide chemotherapy in the mid 20th century. The discovery of the antibiotic penicillin is attributed to Alexander Fleming, but it was isolated by Florey and Chain. Since then there has been enormous number of antibiotics with a wide range of anti bacterial activity.

The theory in which the quantity of bacteria in a wound established a potential for infection was generated by French surgeons[34] during World War I. [ ] If the wound exceeded 15 hours, then wound was debrided, a culture was taken, irrigation with Dakin's solution (Sodium hypochlorite) was instituted and the wound was packed with flavine gauze. The soldier was then referred to a tertiary care hospital where the plate was inspected. If there were no streptococci on the plate or if other bacteria were present at a number less than 5 colonies the wound was sutured after the pack was removed. If it in the reverse state then the wound was allowed to remain open and to close by secondary intention. This early wound therapy not only differentiated between species differences in virulence but concomitantly substantiated the hypothesis that quantitative assessment of the microbial contamination of the wound was invaluable.

The technique of quantitative wound bacteriology was applied in various conditions especially in the management of acute and chronic nonhealing wounds. One of the important clinical aspects of the quantitative bacteriology is the comparison of results of skin grafting shows that 94% Graft survival present when the wound biopsies were determined to be less than or equal to 10^5CFUs per gram of tissue and those that had higher counts had less than 20% graft survival only[49].

CHRONIC NON HEALING WOUNDS:

Chronic wounds can impart an enormous burden on the patient, the health care provider and the health care systems. Most chronic wounds are associated with chronic venous stasis, diabetes mellitus, and pressure necrosis. These wounds cause a major disability and are characterized by chronicity and frequent relapse; they have a significant impact on the socioeconomic

wellbeing of the population and attract enormous health care expenditures. In chronic wounds the healing process is prolonged and incomplete, proceeding in an uncoordinated manner which results in poor anatomic and functional outcome.

IMPAIRED WOUND HEALING:

The systemic factors detrimental to wound healing are ageing, smoking, malnutrition, obesity, diabetes, immunodeficiency status and certain medications. Wound infection is implicated most commonly as a cause for poor wound healing among the local factors like tissue hypoxia or the presence of necrotic tissue. Patients with normal immune system can heal wounds contaminated with up to 100,000 organisms per gram of tissue; bacterial inoculum greater than this are capable of causing clinical wound infection. Infection prolongs the inflammatory phase of wound healing causing the secretion of proteases that degrade newly formed granulation tissue and preclude the wounds ability to progress in to the maturation phase. Impairment of oxygen delivery to the injured tissue also can delay normal wound healing by reducing the production of collagen and its cross linking. Devitalized tissue promotes poor wound healing by acting as a culture medium for bacteria. Furthermore, endotoxin is exuded from the necrotic tissue which can inhibit the migration of fibroblasts and keratinocytes. Systemic disease such as diabetes mellitus contributes to poor wound healing by impairing the wound's ability to control bacterial load because of the glycosylation of neutrophils and macrophages. Diabetes also can contribute to ischemia in the wound leading to hypoxia in the wound bed. Malnutrition can have a detrimental effect because the high metabolic demands of the healing wound cannot be met and it impairs host immunity.

THE ROLE OF MICROBES IN CHRONIC WOUNDS:

Open wound pathogens are commonly considered to be aerobic, (essentially Staphylococci and Streptococci species) [3]. But anaerobic species are also now thought to have a role to play because the frequency of their isolation increases in clinically infected chronic wounds. A third group of organisms, gram negative bacteria[3] (eg.Pseudomonas aeruginosa, Escherichia coli, Klebsiella, Proteus, Acinetobacter and Enterobacter species) tend to appear in the open wound at approximately 4 weeks from initiation. This group generally does not penetrate, but adds to the large numbers of organisms in the wound bioburden. Gram negative bacteria possess antiphagocytic and adherence mechanisms, endotoxins and some exotoxins making them difficult to remove and kill and allowing the toxins to prolong the inflammatory response into a chronic disordered process. Pseudomonas exotoxin pyocyanin can cause wound extension without cellulites. Hence chronic infected wounds are polymicrobial and of mixed aerobe/anaerobe populations, making it impossible to designate the pathogens. Although competition through cohabitation on intact skin appears to decrease the virulence of an individual species. The polymicrobial nature of the open wound is likely to provide opportunities for synergism, producing infection or delayed healing.

Another consideration is the effect of specific species on the wound. Beta hemolytic Streptococci, notably (Streptococcus pyogenes), are pathogenic at numbers that are significantly lower than many other species. Trengrove[36] et al support the notion that the presence of multiple species (four or more) delays healing.

BACTERIAL BIO BURDEN AND BIO FILM:

Four basic categories exist in open wounds resulting from the level of bioburden present based on the induced host response. These categories are defined as contamination, colonization, Local infection (critical colonization) and spreading infection. The last two categories have the potential to disrupt the orderly healing sequence, which results in the development of a chronic wound.

Contamination: is defined as the presence of non replicating microorganisms within a wound. Most organisms entering the wound fall in to this category and are incapable of replicating in soft human tissue. As a consequence, the host defenses rapidly clear them.

Colonization:

Colonization is categorized as replicating microorganism that adhere to the wound surface but do not cause cellular damage to the host. [79]

Critical colonization:

It's a novel concept that states that the bacterial burden in chronic wound does not elicit typical signs and symptoms of infection but delays healing[79]. White and Cutting[91] proposed that this occurs because the bacteria in the wound do not incite an intense inflammatory response through the production of proteins that make them evade the immune system effectively; thus the classic signs of infection are absent, but there is delayed healing through the inhibition of key cells in healing or by the presence of biofilms. Diagnosis of critical colonization is made from two main signs: cessation / delay in healing (despite receiving what would normally be considered

effective therapy) and the absence of cellulites. In addition, corroborative signs include a wet rather than moist wound, abnormal smell, change in exudates color, dull dark red or overtly bright red discoloration of granulation, a pale edematous wound base that does not have a granular appearance and more pain or different pain than usual.

Bio film:

Bio film are complex communities of bacteria that have evolved ways to communicate with each other through water channels and have a protective extra cellular polysaccharide matrix covering. [83] Through these communication channels, the bacterial colonies are able to up regulate or down regulate transcription of genes and protein products that are beneficial to them and detrimental to the host by a phenomenon called quorum sensing. Bio film has high resistance to antibiotic. Bacterial biofilms have been reported from isolates taken from chronic wounds. [46, 62]

Clinical infection:

The presence of multiplying bacteria in the body tissue that results in spreading cellular injury as a result of toxin, competitive metabolism and inflammation. The cardinal features of an infection such as heat, swelling, surrounding erythema and pain are still the standards by which infection is diagnosed; however in chronic wounds distinguishing a true infection from colonization often is difficult. So while dealing with chronic wounds, increasing ulcer size, increasing exudates and friable unhealthy granulation tissue also should be taken in to account. [86]

In view of increasing antibiotic resistance, we are faced with the challenge of antibiotic use. When then, does the presence of bacteria in the

wound become a deter rant to healing? All chronic wounds are colonized with bacteria, this is due in part to the fact these wounds remain open for prolonged periods but is also related to other factors such as poor blood flow, hypoxia and the underlying disease process.

A critical Bacteria load, the synergistic relationship between microorganisms, presence of specific pathogens is the adverse factors which affect wound healing.

Based on various studies, it is inferred that regardless of the type of microorganism, impairment of wound repair may occur when there are more than 1x 10^5 colony forming units per gram of tissue. [79, 87, 91]It has been also suggested that presence of 4 or more type of organism is a predictor of impaired healing, possibly due to synergistic action of microorganisms. The presence of ß hemolytic streptococci in the wound delays wound healing.

MICROBIOLOGICAL ANALYSIS OF CHRONIC WOUND INFECTIONS:

Diagnosis of chronic wound infection based on clinical signs and symptoms alone is difficult. Regular sampling of the chronic wound either by surface swab or tissue biopsy for culture is also done to monitor for the presence of infection. Quantitative culture of tissue biopsy samples and histological verification of microbial invasion into viable tissue have been the "gold standard" for confirming the presence of invasive wound infection,

Wound Sampling Techniques: A variety of different approaches have been described for assessing the nature and extent of microbial involvement in wounds, although the optimal sampling technique continues to be debated.

Superficial wound samples. Clinical microbiology laboratories routinely provide semi quantitative or qualitative results from cultures of superficial wound samples. A number of techniques for the collection of chronic wound surface cultures have been described over the last several decades;

Wound surface swabs are a convenient and effective method for routinely collecting multiple superficial wound samples.[12] In order to obtain enough cellular material for culture, the end of a sterile swab is moved over a minimum 1-centimeter area of the open wound. Sufficient pressure should be applied to the tip of the swab to cause minimal bleeding in the underlying tissue. Evaluations of the recovery of organisms using both dry and moistened swabs have shown that the moist-swab technique provides better reproducibility. [15]

Capillarity gauze sample[91] collections are done by applying gauze squares moistened in nonbacteriostatic saline to the open wound surface for several minutes, followed by use of the contaminated surface of the gauze to inoculate agar culture plates[13]

Absorbent disc technique described by Bruce Williams[95] is the refinement of gauge capillary method.

Agar contact plates[72] may also be applied directly to the open wound surface, but this method has not been adopted into clinical practice because it is the least reproducible sampling technique, and culture medium sterility is not easily maintained outside of the microbiology laboratory.

The quantitative wound biopsy culture method was widely adopted into practice following the studies by Loebl and colleagues[57] After the wound

surface is cleansed with normal saline, two parallel incisions are made in the skin approximately 1 to 2 cm in length and 1.5 cm apart. Sterile tissue forceps are then used to elevate and biopsy a sample with a sterile scalpel from the subcutaneous tissue at sufficient depth to obtain a small portion of the healthy underlying fat. Biopsy samples may also be collected by 3-mm punch biopsy. Tissue biopsy samples obtained by this method typically weigh between 0.02 and 0.05 g. Biopsy specimens are then placed on a nonbacteriostatic moistened sterile gauze pad within a sterile container in order to prevent tissue sample desiccation during transport. Other investigators have also shown that quantitative wound biopsy cultures are more accurate than superficial surface cultures for diagnosing invasive infection in wounds.[10] Disadvantage of biopsy method is that it is invasive, needs surgical preparation.

Another technique involving dermabrasion has recently been described that enables the acquisition of deeper tissue without being as invasive as the biopsy method. [68]

Wound fluid Sampling

When a copious volume of wound fluid exists, sampling by needle aspiration can be employed. This is the most useful procedure for sampling purulent fluid from intact cutaneous abscesses. In cavity wounds such as some pressure sores, irrigation with sterile saline and gentle massaging may be performed to provide fluid for aspiration.

Specimen Transport:

Following the acquisition of wound fluid or tissue for microbiological analysis, prompt delivery of the specimen to the laboratory is considered to be of utmost importance particularly if anaerobic bacteria are being investigated.

Aspirates of purulent fluid and tissue samples are considered to be preferable to swabs[91] because they will maintain the conditions required to sustain microbial viability (i.e. a moist and reduced environment) if processed promptly. However pre reduced commercially available transport media offer advantages if specimen culture is delayed beyond 1-2 hours after isolation.

Analysis of Wound Specimens:

Information regarding the type of wound (e.g. Diabetic, Venous leg ulcer or pressure ulcer), position of the wound, clinical signs of infection, presence of necrosis, associated malodor, and antimicrobial therapy will greatly assist the microbiologist in predicting the microorganisms that are most likely to be involved and therefore the types of culture media and complementary analysis that should be used. Also, the provision of information regarding current antibiotic treatment may assist the microbiologist in determining which microorganisms are most likely to persist in a wound and therefore guide appropriate culturing procedures.

Gram Stain

Despite being used for over a century Gram's stain is still the most important stain in microbiology. In wound management, Gram staining of a known volume (0.2 ml) of tissue biopsy specimen homogenate is spread over a glass slide and dried at 45ºC for 15 minutes and the presence of single organism in the entire field under 1000x is regarded as equivalent of the apparently critical level of 10^5 bacteria per gram of tissue has been used to rapidly estimate the microbial load of a wound and thus facilitate successful closure of surgical wounds.[35] Disadvantage of this Slide smear technique is its dubious accuracy.

SEMIQUANTITATIVE BACTERIAL CULTURE ASSAY:

Wound sepsis is an important problem for chronic non-healing wounds. Evaluation of this condition has been extremely difficult because exposed wound surfaces are readily available for bacterial colonization. It has been recognized that the degree of bacterial wound contamination has a direct relationship with the risk of wound sepsis. Loebl et al[57]. described a quantitative culture technique (Q technique) of full-thickness eschar biopsy that was useful in prediction of burn wound sepsis, a major cause of death among severely burned patients. Observations indicated that patients whose biopsies contained more than 10^5 CFU per g of tissue were likely to develop wound sepsis[57,71] Other investigators have successfully applied similar Q techniques to determine graft bed receptiveness and to predict the safety of wound closure[73,74] Although the degree of bacterial contamination is important in the prediction of wound sepsis, it is necessary to remember that the presence of Streptococcus pyogenes in any numbers constitutes a serious threat to the patient and must be detected by whatever culture method is used.

The Q culture has been recognized as a valuable index for surgeons in the management of severely burned or chronic non healing wound patients, but this culture technique is both labors intensive and expensive. In an effort to reduce the expense of the culture while still providing vital information to the physician, the accuracy and usefulness of a semi quantitative (SQ) biopsy culture was evaluated. In a study by Kay Buchanan[42] et.al 78 eschar biopsies were cultured by a semiquantitative technique that involved the use of 0.1- and 0.01-mI samples of inocula and by the serial dilution method. Exact colony counts from the semiquantitative culture method were available only from cultures containing 10^4 to 10^6 CFU/g of tissue. Other colony counts were

reported as less than 10^4 or greater than 10^6 CFU/g. Agreement by category of colony counts between the two methods was 96%. For prediction of wound sepsis, the semi quantitative procedure had a positive predictive value of 100% and a negative predictive value of 93.7%. This method also resulted in an approximately 30% reduction of work units (as defined by the College of American Pathologists) and a 60% reduction in the amount of media for specimen processing. Therefore, this semi quantitative culture technique provides accurate information to the physician while saving both time and materials.

Culture of wound specimens and Antibiogram:

Routine analysis of wound specimens normally involves the use of selective and non selective agar media to culture aerobic bacteria and yeasts and if a specimen is purulent and or malodorous, anaerobic bacteria also. Although anaerobic bacteria often constitute a significant proportion of the total microbial flora in wounds, their culture and isolation is prolonged and more resource demanding than investigations of aerobic bacteria, and consequently, anaerobic microbiology is often excluded from a routine analysis. Following incubation under aerobic or anaerobic conditions for 24 to 48hours, qualitative and semi quantitative assessments of the cultures are normally made. Antibiograms are most frequently performed for the aerobic pathogens, particularly if they are cultured in abundance and with minimal cohabiting micro flora. If aerobes are absent, but the wound is reported as being clinically infected, anaerobes should be suspected and investigated more thoroughly.

The control of wound infections has become more challenging due to widespread bacterial resistance to antibiotics and to a greater incidence of infections caused by extended spectrum beta lactamase (ESBL) producing

strains, Methicillin Resistant Staphylococcus aureus, polymicrobial flora and by fungi.

ANTI MICROBIAL TREATMENT OF CHRONIC WOUNDS:

The routine use of antibiotics to facilitate wound healing is not supported by evidence. The study by O' Meara[66] et al concerning systemic antibiotics was that insufficient evidence for their use in wound healing. Hutchinson[38] et al. Were also unable to find sufficient information to determine whether antibiotics are more effective than placebo for superficial or deep skin ulcers. The clinical guidelines on Type 2 diabetes by Hutchinson[38] et al recommend only that ulcers with extensive cellulites and/or osteomyelitis should be treated with intensive systemic antibiotics. The UK National Institute for Clinical Excellence[64] in 2004 recommending only that patients with non-healing or progressive ulcers with clinical signs of active infection receive intensive systemic antibiotics. The Scottish Intercollegiate Guidelines Network56 (SIGN) for chronic leg ulcers recommending that systemic antibiotics only be instituted when there is clinical evidence of infection.

Antibiotic use in Clinical practice:

Despite the scarcity of evidence supporting the effectiveness of antibiotics, they are still widely used in the chronic wounds. In the UK, investigation of the prescription of antibiotics for chronic wounds of all aetiologies in the community using the General Practice Morbidity Database for Wales (GPMD) found that Chronic wound patients received significantly more antibiotics than matched non-wound patients.

Topical antimicrobials in wound care:

The benefit of topical antimicrobials may, theoretically, be due to their ability to deliver high local concentrations of antibiotic irrespective of vascular supply[56]. Further benefits which have been cited include the avoidance of adverse systemic effects, and a low incidence of resistance However, others argue that topical antibiotics are a major driving force behind the development of antibiotic resistance. There are also concerns regarding toxicity to human cells, and sensitization, the incidence of which varies considerably between substances.

Antibiotic resistance and chronic wounds:

The combination of increasing numbers of the population who are at risk of developing chronic wounds, together with the increasing prevalence of antibiotic resistance, makes this a highly pertinent issue. The polymicrobial nature of chronic wounds is likely to provide an appropriate environment for genetic exchange between bacteria. Indeed, the first two cases of vancomycin resistant S. aureus in the United States were both isolated from chronic wound patients[16] It is hardly surprising that antibiotic-resistant organisms have been found to colonize and infect chronic wounds. Colsky et al[19] found as many as half of all S. aureus isolates from hospitalized dermatology patients with leg ulcers to be methicillin-resistant S. aureus (MRSA) and more than one-third of P. aeruginosa isolates to be resistant to ciprofloxacin. A prospective study of uninfected chronic venous leg ulcers from 66 patients who had received no antibiotics in the previous month identified very low levels of antibiotic resistance; only two patients were found to have MRSA [7.7% of those patients colonized with S. aureus (n = 26)]. Day & Armstrong[22] reviewed the risk factors for the carriage of MRSA in diabetic foot wounds and suggested risks

include cross-contamination of wounds from the patients themselves, inanimate objects or health care personnel, long-term use of antibiotics, prior hospitalization and severity of illness (which may increase exposure to MRSA endemic environments, such as hospitals and nursing homes). The risk that wound patients carrying antibiotic-resistant organisms pose to others is also unknown. However, dressing changes alone have been shown to disperse significant numbers of bacteria into the air.[27] The extent of this dispersal varies according to the type of dressing involved and is slow to decline. Wound patients are also clearly a group of patients who have a high level of contact with health care staff and could themselves act as a reservoir for cross-contamination. Overall, the morbidity, mortality and cost associated with infections in hospital patients caused by antibiotic-resistant organisms has been shown to be 1.3- to 2-fold higher than infections caused by antibiotic-sensitive organisms.It is clear from the literature that expert opinion suggests that antibiotics have an important role to play in the treatment of clinically infected chronic wounds. However, there are no conclusive scientific studies to support antibiotic use, let alone those that might definitively guide antibiotic choice, dose and duration. Antibiotic resistance in the general population is a continuing and growing concern. The contribution made to the development, maintenance and dissemination of resistance by those antibiotics issued for chronic wounds is not yet known, although there is reason to believe that the chronic wound patient population may be of importance due to the high levels of antibiotic prescribing to these patients, the degree of microbial load associated with their lesions and the potential they provide for dissemination of resistant organisms to others. MRSA and other resistant organisms have been isolated from both infected and colonized chronic wounds.

Alternative antimicrobial therapies. The need for identification and development of new antimicrobial agents that are safe and broadly effective and have a low propensity to induce resistance becomes increasingly critical. In recent years, widespread interest has focused on a class of naturally occurring peptides that protect a variety of animals from infection. These peptides are found in a variety of cell types and operate by attaching to microbial cells, perforating the cell wall, and inducing leakage of cell contents. Many essential oils possess antimicrobial properties, and tea tree oil in particular (derived from the Australian native plant (Melaleuca alternifolia) has been recognized for its efficacy against methicillin-resistant S. aureus and has consequently been considered as an alternative treatment for mupirocin-resistant methicillin-resistant S. aureus[14]. Both honey and sugar pastes are considered useful as topical antimicrobial agents, primarily as a consequence of their high osmolarity and ability to minimize water availability to bacteria[61]. The slow and sustained production of hydrogen peroxide by some types of honey (e.g., manuka honey) is capable of maintaining an antimicrobial effect at a concentration approximately 1,000-fold lower than that commonly used in antiseptics. (i.e., 3%) Also, components of manuka honey such as flavonoids and aromatic acids, demonstrate antimicrobial properties. Honey is also an effective wound deodorizing agent.

OPTIMIZE THE NON-HEALING WOUND FOR SURGERY:

Wound bed preparation offers opportunities for the management of wounds. There are four components to wound bed preparation, which address the different pathophysiological abnormalities underlying chronic wounds. Based on the work of the International wound Bed Preparation Advisory Board, an acronym has been formed. In a recent position document titled

Wound Bed Preparation in Practice, Falanga[33] emphasized the acronym **TIME**, which addresses the 4 components of wound bed preparation: **T**issue management, **I**nflammation and *I*nfection control, **M**oisture balance, and **E**pithelial (**E**dge) advancement. Although none of these components can be singled out as being more important than the other, reducing the bacterial burden and thereby allowing chronic inflammation to subside and preventing infection is clearly recognized as an important requirement in the management of chronic wounds.

MECHANICAL ADJUNCTS IN WOUND HEALING:

Chronic or non-healing wounds demand an aggressive, multifactorial approach including surgical debridement, revascularization, antibiotics and dressings. In addition several adjuvant treatment methods have been developed to further stimulate healing. They include Hydrotherapy, Ultrasound, Negative Pressure therapy, hyperbaric oxygen therapy and Electrical Stimulation. Among these, Electrical stimulation may be one of the upcoming therapies for the future. [36]

ELECTRICAL STIMULATION

Over 25 yrs many controlled research studies have produced evidence that low voltage electric current leads to augmented and accelerated wound healing. Increase rate of new bone formation was demonstrated when small direct current was applied in fracture site. How ever the healing rate of soft tissue wounds particularly superficial open wounds have benefited by addition of electrical stimulation to the treatment regime.

SKIN BATTERY

Transcutaneous potential differences exist in normal human skin is known as the skin battery. The stratum corneum is negatively charged with respect to deeper dermis with an average potential difference of 23 volts. It's believed that these potentials are generated in actively metabolizing basal region of epidermis (stratum basal).A term called "**current of injury**" is generated between the skin and deeper tissue, when there is a break in the skin. This current is expected to continue until the skin defect is repaired. Healing of injured tissue is arrested (or) incomplete if the current no longer flow adequately. The rationale for applying the electrical stimulation is that it mimics the natural current of injury and it will jump start (or) accelerate the wound healing process.

Types of current:

Several types of current used for wound healing .Direct current, Pulsed current, Alternate current.

Direct Current:-

Low intensity direct current clinically 20-100μA at voltage <8volt are used. The cathode is kept over the wound, the anode is placed in peri wound region and the reversed polarity is used in certain regions.

Pulsed Current:-

PC is the brief unidirectional or bidirectional flow of charged particles (electrons or ions) in which each pulse is separated by a longer off period of no current flow. PC is described by its waveform, amplitude, duration, and frequency. PC can have 2 waveforms:

Monophasic PC waveforms that have been described in the clinical wound healing literature include the rectangular waveform and the twin-peaked waveform of high voltage PC. High voltage PC (HVPC) typically has very short-duration (2-20 μs) twin triangular pulses that have single-phase charges on the order of 1.6 μQ.

The biphasic PC wave form also represents a very brief duration of movement of electrons or ions. However, in this case, the pulse is bidirectional and consists of 2 phases. The biphasic waveform may be asymmetric or symmetric about the isoelectric line. In the symmetric biphasic waveform, the phase charges of each phase are electrically equal or balanced; therefore, there is no polarity. Asymmetric biphasic waveforms may be electrically balanced or unbalanced.

Alternate current:-

AC is the continuous bidirectional flow of charged particles in which a change in direction of flow occurs at least once every second.

With so many variables in terms of delivery there are obviously a number of different approaches possible for electrical stimulation of chronic wounds. This makes it difficult to compare individual studies. There is, however, a large body of clinical evidence (Ojingwa[65], 2003) to indicate that healing is improved by E-stim regardless of the type of current applied and experimental evidence to identify possible mechanisms of healing stimulation.

Effects of Electrical stimulus[27,47,65]

Inflammatory phase

Initiate the wound repair by its effect of a current of injury/Increased blood flow/Promote phagocytosis/Enhances tissue oxygenation/Reduces edema

perhaps from reduced micro vascular leakage/Attracts and stimulates fibroblast and epithelial cell/Stimulates DNA synthesis/Controls infection (proven bactericidal effect)/Solublizes blood products including necrotic tissue.

Proliferation Phase

Stimulates fibro blast & epithelial cells/Stimulate DNA and protein synthesis/Increases ATP generation/Improves membrane transport/Produces better collage matrix organization/Stimulate wound contraction

Epithelization Phase

Stimulate epidermal cell reproduction and migration/Produces smoother and thinner scar.

HOW ELECTRICAL STIMULATION AIDS HEALING:

The interaction of E-stim with the chronic wound to initiate the healing effects described earlier has recently been reviewed in detail (Cutting[21], 2006). Many functional cellular defects are known to be associated with the non-healing state of chronic wounds such as bacterial bioburden,chronic inflammation, defective granulation tissue and defective re-epithelialisation that causes a slowing or cessation of healing. E-stim has been shown to inhibit bacterial growth in vitro (Kincaid & Lavoie[45], 1989) and on intact human skin. Anti-bacterial effects have been demonstrated in P. aeruginosa infected experimental incisional wounds (Rowley[75] et al., 1974) and similarly bacterial proliferation within human pressure ulcers has been demonstrated to be inhibited after three days of E-stim treatment (Wheeler et al., 1971). E-stim is known to disrupt biofilms (Costerton[23] et al., 1994) and the recent successful treatment of a leg ulcer where healing was delayed by biofilm suggests that this E-stim delivery by Low voltage Pulsed current acts to disrupt bacterial biofilms.

The mechanism of the antibacterial activity of electrical current has been suggested to result from toxic substances (H2O2, oxidizing radicals and chlorine molecules) produced as a result of electrolysis[89], the oxidation of enzymes and coenzymes, membrane damage leading to the leakage of essential cytoplasmic constituents and/ or a decreased bacterial respiratory rate. Decreasing the number of bacteria within chronic wound tissue will assist in conversion of chronic inflammation to a resolving inflammatory response. Formation of healthy granulation tissue depends on the proliferation of fibroblasts and their ability to synthesise a functioning extracellular matrix. There is considerable experimental evidence that E-stim interacts in all aspects of granulation tissue synthesis. It increases fibroblast protein synthesis and proliferation, increases collagen production and improves collagen fibre organization to give increased wound strength. Angiogenesis can be enhanced by E-stim improving dermal capillary formation in human ischemic wounds possibly by stimulating angiogenic responses after interacting with endothelial cell growth factor receptors. Once a healthy and functional wound bed has formed keratinocytes have to migrate over it to close the wound and form new epidermis. In the same way that macrophages will migrate towards the cathode in electrotherapy systems keratinocyte migration is also enhanced and directed in the same way. The effect of enhanced directional migration in an electrical field is called galvanotaxis or electrotaxis and plays an important role in the healing process.

Clinical evidence for efficacy of electrical stimulation: Leg Ulcers*:* Low voltage DC E-stim is effective in accelerating healing of leg ulcers where a 2.5 times faster healing rate was observed compared to standard wet-to-dry dressings and whirlpool therapy. The E-stim treated ulcers also

required less debridement, no infections occurred and the patients reported less pain at the wound site. In a double blind prospective study of 27 subjects 42 ulcers of venous, arterial or diabetic origin (Houghton et al., 2003) [65] were randomized to two groups that received E-stim or sham treatment for 45 minutes three times a week for four weeks. The investigators concluded that 'E-stim should be used to accelerate healing for chronic vascular ulcers' as ulcers in the active treatment group reduced in area by approximately 50 per cent over the four week treatment period. The Bio-electric wound dressing[32] has a circular anode that contacts the peri-wound skin and a central cathode that is placed in contact with the wound bed. A DC micro current generated by an integral miniature circuit and battery passes between the electrodes to replicate the natural current of injury and stimulate healing .E-stim with this LVPC dressing may stimulate healing, at least in part, by exerting an anti-bacterial effect. Another non-healing venous leg ulcer of two years duration in which bacterial biofilm was judged to be delaying wound healing was also induced to initiate healing (White et al., 2006).

MATERIALS AND METHOD

STUDY TYPE:

Randomized controlled Prospective study.

STUDY PLACE:

This study was done in department of Microbiology, Stanley medical college, in association with department of plastic surgery, Govt.Stanley hospital Chennai.

STUDY PERIOD:

June 2008 to May 2009.

STUDY POPULATION:

During this study period, patients with chronic non healing superficial wounds admitted to the department of Plastic surgery with various etiologies were included. Among the 38 patients selected, 36 patients had single ulcer and 2 patients each had two ulcers. All these ulcers were mostly present in the lower limbs except two ulcers which were present in the upper limbs. Out of the 40 wounds from 38 patients, 20 wounds were kept randomly in the study group and another 20 wounds were kept randomly in the control group.

Study group- Electrical stimulation treatment was given for a period of 3 weeks.

Control group **-** Without electrical stimulation only saline dressing was given for 3 weeks.

None of the group was treated either with antibiotics or topical antiseptics.

INCLUSION CRITERIA:

Patients with chronic superficial non healing wounds of varying etiology like diabetes, arterial, venous and pressure ulcers which do not heal in the expected time frame(about a month time) with conventional treatment like optimization of nutrition, moist dressings, debridement, infection resolution, repositioning, and off-loading of pressure.

Patients of either sex with ages as and above 15 years

Patients who gave written informed consent to undergo this treatment modalities and various investigations.

Patients who were not on systemic and topical antibiotics or antiseptics during this study period.

EXCLUSION CRITERIA:

- Patients on either systemic and topical antibiotics or antiseptics.
- Malignant ulcer
- Any indication of thrombosis underlying the wound.
- Chronic Osteomyelitis
- Patients using cardiac pacemaker were excluded from the study.
- Pregnant woman

SPECIMEN COLLECTION:

Four tissue specimens were taken at timely interval from each chronic non healing wounds of both study as well as control groups. First sample was taken before starting electrical stimulation. Second and third samples were taken at weekly interval after starting electrical stimulation. Fourth sample was

taken at the end of third week after finishing electrical stimulation. Similarly for the control group wounds also 4 specimens were taken before and after saline dressing treatment.

Method of Wound Biopsy:

The wound area was first thoroughly washed with sterile normal saline. By using a sterile punch biopsy forceps the tissue bits were collected in a sterile test tube to which small amount of sterile saline was added to keep the specimen moist and brought to the dept of Microbiology with proper labeling and clinical details.

SPECIMEN PROCESSING:

1. After careful decanting of the saline, the tissue bits were transferred in to a pre weighed 20ml sterile beaker using sterile glass rod and weighed in an electrical balance and tissue weight was calculated.

2. The tissue specimen was homogenized well with the 2ml of sterile nutrient broth by using an autoclaved porcelain mortar and the pastel.

Slide smear technique for quantitative assessment: After homogenization, 0.2 ml of tissue homogenate was applied on a clean glass slide and was spread as a thin smear. It was allowed to air dry for 15 minutes. After the slide was heat fixed Gram staining was done. Then the stained direct smear was examined with 100x oil immersion objective. The presence of a single organism in the entire field was regarded as equivalent of the apparently critical level of 10^5 bacteria per gram of tissue. Gram stain morphology and presence of any pus cells were documented.

Semi quantitative bacterial culture assay streaking technique:

From the tissue homogenate, a 0.1 ml sample was inoculated to a blood agar plate with a micro pipette. Additionally, a 0.01 ml sample was inoculated on another BA plate. This method was done in duplicate to assess the

reproducibility of colony counts obtained by this procedure. In this method first the required volume of inoculum was delivered to the surface of an agar plate by making a single streak across the centre. Then without flaming the loop the inoculum was spread evenly at right angles to the primary streak, then the plate was turned 90 degrees and the inoculum was spread to cover the entire surface to produce isolated colonies.

In addition to BA plates, 0.01 ml of homogenate was inoculated on to a MacConkey agar plate as a differential medium. After plating, the Petri dishes were incubated aerobically at 37ºC for 18-24 hrs.On the second day the plates were examined.

Interpretation of Semi quantitative Bacterial Culture Assay:

Exact colony counts were obtained from semi quantitative assay as like in the Quantitative assay from BA plates with 30 to 300 colonies by using a Colony counter. If there were more than 300 colonies obtained on both plated dilutions, the factor 300 was used as N for calculations, and the result was considered greater than the value.The number of CFUs per gram of tissue was calculated by applying the following formula:

Number of CFUs counted x Reciprocal of volume of homogenate inoculated (10^{-1} or 10^{-2}) x 2 (volume of diluents used for tissue homogenization) divided by the weight of tissue in grams.

Semi quantitative assay results were reported as the following responses:

(I) No growth, (ii) less than 10^4 CFU/g of tissue, (iii) more than 10^6 CFU/g, or (IV) the exact colony counts in the range 10^4 to 10^6CFU.

Qualitative bacteriology:

Organisms were identified by colony morphology, Gram's staining, and motility and biochemical reactions. Antimicrobial susceptibility testing was carried out by using the Kirby Bauer disc diffusion method.

GPC were identified on the following media as follows

Media	Colony morphology	Gram stain	Probable organism	Tests done
Blood Agar	Convex, entire edge, 2-3 mm, creamy, yellowish / whitish, zone of β hemolysis	GPC in clusters	Staphylococcus species	-Catalase -Coagulase -DNase -Mannitol utilization -Tellurite reduction -Novobiocin resistance Furazolidone resistance
Blood Agar MacConkey Agar	Non hemolytic colonies Magenta coloured colonies	GPC in pairs, short chains	Enterococcus species	a)Heat test b)Bile esculin hydrolysis

Differentiating characters of gram negative bacilli for their identification were as follows:

Organism	Motility	Catalase	Oxidase	Indole	MR	VP	Citrate	TSI	Urease	PAD	Glucose	Lactose	Sucrose	Maltose	Mannitol
Escherichia coli	+	+	–	+	+	–	–	A/A Gas	–	–	+	+	–	+	+
Klebsiella pneumonia	–	+	–	–	–	+	+	A/A gas with gas	+	–	+	+	+	+	+
Klebsiella oxytoca	–	+	–	+	–	+	+	A/A gas	+	–	+	+	+	+	+
Citrobacter freundii	+	+	–	–	+	–	+	K/A A/A gas& H2S.	+/–	–	+	±	+	+	+
Citrobacter koseri	+	+	–	+	+	–	+	K/A,gas	+/–	–	+	±	±	+	+
Proteus vulgaris	+	+	–	+	+	–	V	K/A ,Gas &H2S	+	+	+	–	–	+	–
Proteus mirabilis	+	+	–	–	+	–	V	K/A gas &H2S	+	+	+	–	–	–	–
Morganella morganii	+	+	–	+	+	–	–	K/A,gas	+	+	+	–	–	–	–
Pseudomonas aeruginosa	+	+	+	–	–	–	+	K/NC	–	–	–	–	–	V	V
Acinetobacter species	–	+	–	–	V	V	–	K/NC	–	–	–	–	–	–	–

Antimicrobial sensitivity testing:

Disc susceptibility testing of the aerobic isolates was performed by modified Kirby Bauer disk diffusion Method in Mueller Hinton agar medium. 25ml of medium was poured in a Petri dish of 90mm diameter to obtain a thickness of 4mm of media.

Preparation of 0.5 McFarland's Turbidity Standard for inoculum preparation:

0.05ml of 1% solution of anhydrous Barium chloride was added to 9.95 ml of 1% cold solution of pure sulphuric acid in a test tube with constant stirring to maintain a uniform suspension. The Barium sulphate suspension was transferred in 4-6 ml to a screw capped tube of the same size as those used in growing or diluting the bacterial inoculum.The tube was tightly sealed and stored in refrigerator. Before each use it was shaken vigorously until all the deposit was raised into uniform suspension. 0.5McFarland's standard which corresponds to 150 million organisms/ml.

Preparation of Inoculum (Growth Method) and Inoculation:

Four to five morphologically similar colonies from an agar medium was touched with a wire loop and the growth transferred to a test tube containing 1.5ml of nutrient broth. The tube was incubated at 35ºC until it matched in density with 0.5 McFarland's standard which corresponds to 150 million organism/ml.Within 15 minutes of preparation of the suspension a sterile cotton wool swab was dipped into the suspension and surplus removed by rotation of the swab against the side of the tube above the fluid level. The medium was inoculated by even streaking of the swab over the entire dried surface of the Mueller Hinton agar plate three times, rotating the plate

approximately 60degrees each time. Finally the rim of the agar was swabbed. The lid of the dish was left ajar for 3 to 5minutes but not longer than 15minutes, for the surface of the agar to dry before placing the antibiotic discs.

Antibiotic discs: The antimicrobial susceptibility testing for Staphylococcus aureus and coagulase negative staphylococcus included penicillin10U, erythromycin15µg, cotrimoxazole 25µg, oxacillin1µg, cefotaxime30µg, ciprofloxacin5µg, gentamicin10µg and amikacin30µg discs and vancomycin30µg disc for oxacillin resistant strains only.

For Enterococci the antimicrobial susceptibility testing included penicillin10U, erythromycin15µg and amikacin30µg discs.

For gram negative bacilli the antimicrobial susceptibility testing included ampicillin10µg, cotrimoxazole 25µg, ciprofloxacin5µg, cefotaxime30µg, ceftazidime30µg, gentamicin10µg and amikacin30µg discs and imepenem10µg disc for ESBL producers only. Enterobacteriaceae isolates with zone inhibition diameter ≤27mm for cefotaxime and ≤22mm for ceftazidime were considered as presumptive ESBL producer by disc diffusion method.

Antibiotic discs were applied with forceps and pressed gently to ensure even contact with the medium. The plates were inverted and placed in incubator at 35ºC to37ºC for 16 to 18hours.

Reading of zones of inhibition:

The diameters of the zones of complete inhibition (as judged by the unaided eye) were measured, including the diameter of the disc. Zones were measured to the nearest whole millimeter, using ruler, which was held on the

back of the inverted Petri plate. The Petri plate was held a few inches above a black, nonreflecting background and illuminated with reflected light.

Interpretation

The sizes of the zones of inhibition were interpreted by referring to the NCCLS Table-2 Volume 20; No 1:2000 (Zone Diameter Interpretive Standards) and reported as susceptible intermediate or resistant to the agents that have been tested (Annexure).

Tube method of identification for Slime production by Pseudomonas aeruginosa isolatated from wound biopsy:

Pseudomonas aeruginosa isolated from chronic wound which had clinical evidence of biofilm formation which appeared as a "glaze' on the surface of wound was subjected for the tube method.

Two to three colonies of P.aeruginosa were inoculated in to 5ml of BHI broth in glass tube. Culture was incubated at 37°C for 18- 20 hours. On the next day the culture contents was aspirated. Then the tube was stained with saffranin. The presence of a visible stained film on the wall of the tube was considered to be positive for slime production.Formation of the ring at the liquid air interface was not considered as a positive test. If the wall of the glass tube remained unstained, the strain was considered as a non slime producer.

CLINICAL PARAMETER:

a) **Clinical examination**

1. Size & shape of the wound / 2. Quantity of exudates / 3. Periwound edema /4. Wound edge /5. Colour of the granulation tissue/6. Perception of pain

b) **Perimetric analysis:**The wound surface area is assessed by tracing the wounds in a transparent sheet.

c) **Photographic analysis:** Serial photographic analysis of the wounds was done in standard positions. The change in size of the wounds could be assessed by comparing the previous photographs.

TREATMENT PROTOCOL:

Every week, the wound appearance (e.g., color, presence or absence of necrotic and/or granulation tissue) was documented and wound surface area was recorded. A color photograph was taken every week to provide a permanent record and for monitoring purpose. The systemic co morbid illnesses like diabetes mellitus, malnutrition, anemia etc are treated accordingly.

STUDY GROUP (ELECTRICAL STIMULATION)

Apart from necessary debridement, the wounds were cleaned with normal saline. Electrical stimulation to the wound was given for 40 minutes daily for a period of 3 weeks.

Type of Current

The electrical stimulation device used in this study was Low voltage pulsed current stimulator (LVPC) which delivered biphasic symmetrical rectangular pulses with an amplitude of 40 mA and at a frequency of 128 pulses per second.

Mode of Delivery

The electrical stimulator has two electrodes, an active electrode and a dispersive electrode. The polarities of the electrodes keep changing alternatively. The frequency of change in polarity depends on the pulse width.

The wound is cleaned and covered with a single layer of moist gauze. An aluminum foil of the same size and shape of the wound is cut and placed over the wound. The active electrode of the electrical stimulator is fixed to the aluminum foil with the alligator clips. The dispersive electrode is kept over moist gauze and placed over the periwound skin 5-10 cm apart and secured safely by bandaging. The current was delivered for 40 mints/day. Everyday after the end of electrical stimulation, electrodes were removed and normal saline dressing was done for the wounds.

No antibiotic or topical antiseptic was given throughout the course of treatment and these wounds were examined at the end of every week as per the treatment protocol.

CONTROL GROUP:

These groups of patients were treated conservatively by saline irrigation and semi occlusive dressing. Debridement was done as and when required. No antibiotic (or) antiseptics was used to manage these wounds. These wounds were also examined as per the treatment protocol.

STATISTICAL ANALYSIS:

The statistical method used in this study was Mann-Whitney/Wilcoxon Two-Sample Test (Kruskal –Wallis Test for two groups). The bacterial counts obtained by Semi quantitative Bacterial culture in CFUs /gram of tissue from study as well as control groups were analyzed as Log transformed data and then it was compared between the groups and these results were correlated with the reduction in wound size and the healing rate. The effect of electrical stimulation was also compared with the control group by the Mean reduction of wound size in Sq.cm with Standard Deviations. Significance was accepted at $P< 0.05$.

ANTIBACTERIAL EFFECTS OF ELECTRICAL STIMULATION: INVITRO STUDY

Materials: The three bacterial species commonly isolated from chronic wounds were used as test organisms. Exponential growth-phase isolates of Staphylococcus aureus (ATCC 25922), Pseudomonas aeruginosa(ATCC 27853) and ESBL Klebsiella pneumoniae(ATCC 700603) were obtained from American Type Culture Collection stocks.

Four autoclavable Petri dishes of 90mm size were selected. Two holes were bored through the bottom of each Petri dish 1.5 cm apart from the centre. Two 7 cm long and 19 G size stainless-steel wires were fed through each hole. A 1.2 cm portion of the electrode inside the Petri dish was perpendicular to the bottom of the dish, while the outside portion was anchored with a quick fix material to the bottom of the Petri dish, with a **2** cm overhang for connecting it to the Electrical stimulator. The Petri dish with the inbuilt electrodes was wrapped in papers individually and they were kept for autoclaving. A custom-made mica switch board was designed to allow stacking of four Petri dishes with their electrodes at a time which were connected to the sachets fixed in the board.The four autoclaved Petri dishes with in built electrodes were connected to the sachets in the mica board without opening the lids and kept in a laminar flow hood after proper labeling.

PROCEDURE:

The selected test cultures which were grown overnight to reach mid exponential growth phase at 37°C in nutrient broth was matched in density with 0.5 McFarland's standard by adding sterile saline to reach a final concentration of 10^8 colony forming units per milliliter of the test organism . This liquid

culture was added to the Mueller-Hinton agar medium. The prepared MHA inoculated with the test organism was poured in to the sterile Petri dish with the electrodes insitu to cover ⅔ of the wire electrodes (4 mm). Then the lid was closed and allowed to solidify. The fourth MHA plate was without any of the test organism and labeled as pH control culture plate .Immediately after solidification the switch board sachets were connected to the instrument via cables.

Another three sets of MHA with the corresponding test organisms with wire electrodes kept as control plates for which no electrical stimulation was given. By observing the control plates without ES, any chemical effect of electrode on the culture can be ruled out.

INSTRUMENTATION:

The electrical stimulation device used in this study was Low voltage pulsed current stimulator (LVPC) which delivered biphasic symmetrical rectangular pulses with an amplitude of 40 mA and at a frequency of 128 pulses per second (pps). Electrical stimulation was given for 2 hrs.

pH control plate was used to determine the pH of the medium after ES. The pH indicator paper strip was applied at 6 min after ES was terminated. This indicator gave a color, showing a range of pH as follows: yellow for a pH of 6.5; yellowish green for a pH of 7; dark green for a pH of 7.5; bluish green for a pH of 8; light blue for a pH of 8.5 and dark blue for a pH of 9.0.

After the ES, the test and control plates were kept overnight in the incubator at 37º C. The next day test plates were screened for the following.

1. Zone of inhibition around the electrodes. Zones were measured to the nearest whole millimeter, using ruler, which was held on the back of the inverted Petri plate. The Petri plate was held a few inches above a black, nonreflecting background and illuminated with reflected light.

2. Each Petri dish was also examined for electrode corrosion, gas formation and media discoloration.

OBSERVATION & RESULTS

During this study period, patients with chronic non healing superficial wounds admitted to department of Plastic surgery with various etiologies were included. Out of 40 wounds from 38 patients (both study and control groups), a total of 160 tissue specimens were collected and processed at the department of microbiology periodically from June 2008 to May 2009. The specimens were subjected for qualitative and semi quantitative aerobic bacterial culture assays and the results were analyzed as follows.

TABLE - 1 : ETIOLOGY OF CHRONIC SUPERFICIAL WOUNDS

(n= 40)

S.No	Etiology of Ulcer	No of Ulcers
1	Diabetic Ulcer	14
2	Arterial Ulcer	10
3	Venous Ulcer	8
4	Chronic Ulcer with lymph edema	6
5	Unstable scar	2

All patients included in the study had wounds in the lower limbs except 2 patients who had wounds in the upper limbs.

On analysis of the etiology of chronic wounds in this study, 14 diabetic ulcers; 10 arterial ulcers; 8 venous ulcers; 6 lymphatic ulcers and 2 unstable scars were present.

TABLE - 2 : AGE AND SEX DISTRIBUTION OF PATIENTS (n = 38)

Age in years	Male	Female	Total
21-30	5	1	6
31-40	6	1	7
41-50	8	2	10
51-60	4	3	7
61-70	4	2	6
71-80	1	1	2
Total	**28(73.7%)**	**10(26.3%)**	**38**

Total number of patients included in this study were 38, among which 28were males (73.7%) and 10 were females (26.3%). Out of them, between 21-30 years of age there were 5 males and 1 female, between 31-40 years 6 males and 1 female, between 41-50 years 8 males and 2 females, between 51-60 years 4 males and 3 females, between 61-70years 4 males and 2 females and between 71-80 years 1male and 1female. Most of the patients were in the age group between 41-50 yrs. The youngest patient was 23 years of age and the oldest was 76 years. Most of the patients with chronic ulcer were males.

TABLE – 3 : RESULTS OF RAPID SLIDE SMEAR GRAM STAIN TECHNIQUE FOR QUANTITATIVE ASSESSMENT

Total No of Direct Smears	Smears Positive for Rapid quantitative assay	Negative Smear
160	77	83

Out of 160 direct smears by Gram's staining, 77smears showed positive screening in the rapid slide smear technique for Quantitative Assessment which was shown to reliably predict a critical microbial load of $>1x10^5$CFUs/ gram of tissue if a single or more micro organism was seen in the entire field under oil immersion objective by using 0.2 ml of tissue homogenate. 83 were reported as negative smear.

TABLE - 4 : CORRELATION OF RAPID SLIDE SMEAR GRAM STAIN TECHNIQUE AND AÉROBIC BACTERIAL CULTURE ASSAY (n=160)

Smear Positive Culture Positive	Smear Negative Culture Positive	Smear Negative Culture Negative
77	47	36

Out of 160 wound specimens, direct Gram staining for quantitative assay revealed 77 were positive smears and 83 were negative smears.By aerobic bacterial culture assay, 124 specimens yielded positive cultures and 36 were no growth.Among the 160 wound specimens, 77 specimens were both smear and culture positive.47 specimens were smear negative but culture positive.36 specimens were negative for both smear and culture techniques.

TABLE – 5 : ISOLATES FROM CHRONIC NONHEALING SUPERFICIAL WOUND SPECIMENS (n=160)

ORGANISMS	CHRONIC SUPERFICIAL WOUNDS										TOTAL n=160
	Diabetic ulcers		Arterial ulcers		Venous ulcers		Lymphatic ulcers		Unstable scar		
	T n=28	C n=28	T n=20	C n=20	T n=16	C n=16	T n=12	C n=12	T n=4	C n=4	
Gram Positive Cocci	12	16	12	15	4	4	4	4	2	4	77 (37.2%)
Staphylococcus aureus	6	12	8	8	4	4	4	4	2	-	52 (32.5%)
CONS	4	-	4	4	-	-	-	-	-	4	16 (10%)
Enterococcus species	2	4	-	3	-	-	-	-	-	-	9 (5.62%)
Gram Negative Bacilli	24	30	12	10	12	12	12	16	2	0	130 (62.8%)
Pseudomonas aeruginosa	5	8	5	4	4	-	4	4	2	-	36 (22.5%)
Klebsiella pneumoniae	11	4	-	-	-	-	-	-	-	-	15 (9.37%)
Klebsiella oxytoca	-	4	-	-	-	4	2	4	-	-	14 (8.75%)
Escherichia coli	-	2	4	6	4	4	-	4	-	-	24 (15%)
Proteus mirabilis	8	-	-	-	-	-	2	4	-	-	14 (8.75%)
Proteus vulgaris	-	4	-	-	4	4	-	-	-	-	12 (7.5%)
Citrobacter freundii	-	4	-	-	-	-	-	-	-	-	4 (2.5%)
Citrobacter koseri	-	-	3	-	-	-	-	-	-	-	3 (1.87%)
Acinetobacter species	-	-	-	-	-	-	4	-	-	-	4 (2.5%)
Morganella morganii	-	4	-	-	-	-	-	-	-	-	4 (2.5%)
Total Isolates	36	46	24	25	16	16	16	20	4	4	207
No growth	9	4	6	1	8	4	2	0	2	0	36

Out of 160 wound biopsy tissue specimens collected from both study and control groups, a total of 207 isolates were obtained. 36 specimens yielded no growth. Among the 207 isolates; 130(62.8%) were gram negative bacilli and 77(37.2%) were gram positive cocci. Among the 207 aerobic isolates, 52(32.5%) were Staphylococcus aureus; 36(22.5%) were Pseudomonas aeruginosa; 24(15%) were Escherichia coli; 16(10%) were Coagulase negative Staphylococcus; 15(9.37%) were Klebsiella pneumoniae; 14(8.75%) were Klebsiella oxytoca; 14(8.75%) were Proteus mirabilis; 12(7.5%) were Proteus vulgaris; 9(5.62%) isolates were Enterococcus species; 4(2.5%) were Citrobacter freundii; 4(2.5%) were Acinetobacter species; 4(2.5%) were Morganella morganii; and 3(1.87%) were Citrobacter koseri .

TABLE -6 : MONO & POLYMICROBIAL INCIDENCE IN CHRONIC NONHEALING SUPERFICIAL WOUND SPECIMENS (n=160)

Type of ulcer		Number of Specimens (n=160)	Positive culture for Bacteria		No growth
			Single Isolate	Two Isolates	
Diabetic	Study	28	--	19	9
	Control	28	4	20	4
Arterial	Study	20	5	9	6
	Control	20	12	7	1
Venous	Study	16	--	8	8
	Control	16	8	4	4
Lymphatic	Study	12	4	6	2
	Control	12	4	8	--
Unstable scar	Study	4	--	2	2
	Control	4	4	--	--
	Total	160	41	83	36

Out of 160 specimens, 124specimens yielded positive culture for aerobes and facultative anaerobes and 36 specimens yielded no growth. Among the 124 positive cultures, 83 specimens yielded two isolates and 41 yielded single isolate per specimen.

TABLE – 7 : POLYMICROBIAL PATTERN IN 82 SPECIMENS

Polymicrobial pattern	Total Numbers
Pseudomonas & Staph.aureus	17
Pseudomonas& others[1]	17
Staph.aureus& others[2]	16
Proteus spp& Klebsiella spp	14
Proteus spp& Escherichia coli	8
Proteus spp & CONS	4
Escherichia coli & CONS	4
Enterococci & Citrobacter spp	3

[1.Klebsiella pneumoniae, Acinetobacter spp, Escherichia coli & Enterococci

2.Escherichia coli, Klebsiella pneumoniae; Citrobacter freundii and Enterococci]

Out of 82 specimens which yielded two isolates per specimen, 17 were combination of Pseudomonas aeruginosa and staphylococcus aureus; in another 17 specimens Pseudomonas aeruginosa was combined with Klebsiella pneumoniae (2), Acinetobacter spp, Escherichia coli and Enterococci; 16 were combination of staphylococcus aureus with Escherichia coli (2), Klebsiella pneumoniae; Citrobacter freundii and Enterococci; 14 were combination of Proteus spp& Klebsiella spp; 8 were combination of Proteus spp & Escherichia coli; 4 were combination of Proteus spp & CONS; another 4 were Escherichia coli & CONS and Enterococci & Citrobacter spp combination were present in 3 specimens.

TABLE – 8 : ANTIBIOTIC SENSITIVITY PATTERN OF STAPHYLOCOCCUS AUREUS AND COAGULASE NEGATIVE STAPHYLOCOCCUS

Antibiotics	Staphylococcus aureus n=52		Coagulase negative Staphylococcus n=16	
	Sensitive	Resistant	Sensitive	Resistant
Penicillin	12(23%)	40(77%)	0	16(100%)
Erythromycin	22(42.3%)	30(57.7%)	0	16(100%)
Oxacillin	33(63.46%)	19(36.54%)	8(50%)	8(50%)
Cefotaxime	41(78.8%)	11(21.2%)	12(75%)	4(25%)
Ciprofloxacin	45(86.5%)	7(13.5%)	12(75%)	4(25%)
Amikacin	35(67.3%)	17(32.7%)	16(100%)	---

Out of 52 Staphylococcus aureus 45(86.5%) were sensitive to ciprofloxacin, 41(67.3%) were sensitive to cefotaxime, 35(67.3%) were sensitive to amikacin. 33(63.46%) were sensitive to oxacillin, 23(44.2%) were sensitive to gentamicin, 22(42.3%) were sensitive to erythromycin, 12(23%) were sensitive to penicillin.

Out of 16 Coagulase negative staphylococcus 16(100%) were sensitive amikacin, 12(75%) were sensitive to cefotaxime and ciprofloxacin and 8(50%) were sensitive to cotrimoxazole, Oxacillin and gentamicin.

TABLE - 9 : MSSA AND MRSA AMONG STAPHYLOCOCCUS AUREUS (n=52)

Methicillin Sensitive SA	33	63.46%
Methicillin Resistant SA	19	36.54%

Among the 52 isolates of Staphylococcus aureus, 19(36.54%) were resistant to 1μg disc of oxacillin by disk diffusion method which indicated that they were MRSA by the preliminary screening test.

TABLE – 10 : ANTIBIOTICS SENSITIVITY PATTERN OF ENTEROCOCCUS SPECIES (n=9)

Antibiotics	**Enterococcus species n=9**
Penicillin	9(100%)
Erythromycin	9(100%)
Amikacin	9(100%)

Out of 9 Enterococcus species were isolated and all (100%) were sensitive to penicillin, erythromycin and amikacin.

TABLE – 11 :ANTIBIOTIC SENSITIVITY PATTERN OF PSEUDOMONAS AERUGINOSA AND ACINETOBACTER SPECIES

Antibiotics	Pseudomonas aeruginosa n=36	Acinetobacter spp n=4
Ciprofloxacin	19 (52.77%)	4(100%)
Amikacin	26 (72.22%)	4 (100%)
Gentamicin	7(19.44%)	0
Cotrimoxazole	0	0
Cefotaxime	0	0
Ceftazidime	15(41.66%)	0

Out of 36 Pseudomonas aeruginosa, 26 (72.22%) were sensitive to amikacin, 19 (52.77%) were sensitive to ciprofloxacin, 15(41.66%) were sensitive Ceftazidime and 7(19.44%) were sensitive Gentamicin. All the isolates were resistant to cefotaxime and cotrimoxazole.

Four Acinetobacter species were isolated and it was 100% sensitive to both ciprofloxacin and amikacin, where as all the 4 were resistant to Gentamicin, Cotrimoxazole, Cefotaxime and Ceftazidime.

TABLE-12 : ANTIBIOTIC SENSITIVITY PATTERN OF ENTEROBACTERIACEAE ISOLATES (n=90)

Antibiotics	Enterobacteriaceae isolates n=90	
	Sensitive	Resistant
Ampicillin	4(4.44%)	86(95.56%)
Gentamicin	20(4.44%)	70(77.8%)
Amikacin	77(85.5%)	13(14.4%)
Ciprofloxacin	35(38.8%)	55(61.1%)
Cefotaxime	51(56.6%)	39(43.4%)
Ceftazidime	51(56.6%)	39(43.4%)

Out of 90 enterobacteriaceae isolates, 77(85.5%) were sensitive to amikacin; 51(56.6%) isolates were sensitive to cefotaxime and ceftazidime. 35(38.8%) were sensitive to ciprofloxacin, 20(22.2%) were sensitive to gentamicin, and 4(4.44%) were sensitive to ampicillin.

39(43.4%) enterobacteriaceae isolates were resistant to both cefotaxime (30µg) and ceftazidime (30µg) which indicated presumptive ESBL producer in the preliminary screening test.

TABLE – 13 : ESBL PRODUCERS AMONG ENTEROBACTERIACEAE (n=90)

Organism	Total	ESBL	Percentage
Escherichia coli	24	12	50%
Citrobacter freundii	4	1	25%
Citrobacter koseri	3	-	--
Klebsiella pneumoniae	15	7	46.66%
Klebsiella oxytoca	14	6	42.85%
Proteus mirabilis	14	6	42.85%
Proteus vulgaris	12	8	66.66%
Morganella morganii	4	-	--
Total	90	39	43.34%

Out of 90 Enterobacteriaceae isolates, 39(43.4%) were found to be ESBL producers by disk diffusion screening method in which they were resistant to indicator cephalosporins [both cefotaxime (30µg) and ceftazidime (30µg)]

Out of 12 Proteus vulgaris isolates 8 (66.66%) were ESBL producers. Out of 24 Escherichia coli isolates 12(50%) were ESBL producers. Out of 15 Klebsiella pneumoniae isolates 7(46.66%) were ESBL producers. Out of 14Klebsiella oxytoca isolates 6(42.85%) were ESBL producers. Out of 14Proteus mirabilis isolates 6(42.85%) were ESBL producers.

Out of 4 Citrobacter freundii isolates 1(25%) was ESBL producer. Citrobacter koseri isolates were three, but they were not ESBL producers.

TABLE – 14 : SEMI QUANTITATIVE AEROBIC BACTERIAL CULTURE ASSAY STUDY GROUP (n=20 WOUNDS) BEFORE AND AFTER ELECTRICAL STIMULATION (ES)

StudyGroup Electrical stimulation (n=20 wounds)		Bacterial Burden in CFUs /gram of tissue		
		Significant Bacterial Count(>1x10^5 CFUs)	Insignificant Bacterial Count (≤10^5 CFUs)	No growth
Before Starting ElectricalStimulation		12	5	3
After starting ES	End of 1st Week	12	5	3
	End of 2nd Week	6	4	10
	End of 3rd Week	1	7	12

Out of the 20 wounds from study group, 12 wounds had significant bacterial count (>10^5CFUs /gm of tissue), 5 wounds had insignificant level of bacterial count (≤10^5CFUs/gm of tissue), and 3 wounds had no growth before starting electrical stimulation treatment. (Chart-1)

At the end of 3 weeks of electrical stimulation, the bacterial count has decreased and only 1 wound had significant bacterial count, 7 wounds had insignificant level of bacterial count and 12 wounds were reported as no growth. (Chart-2)

TABLE-15 : SEMI QUANTITATIVE AÉROBIC BACTERIAL CULTURE ASSAY CONTROL GROUP (n=20 WOUNDS) BEFORE AND AFTER SALINE DRESSING

Control Group Saline Dressing (n=20 wounds)		Bacterial Burden in CFUs /gram of tissue		
		Significant Bacterial Count(>1x10^5CFUs)	Insignificant Bacterial Count (≤10^5 CFUs)	No growth
Before Starting Saline Dressing		11	7	2
After Saline dressing	End of 1st Week	13	5	2
	End of 2nd Week	14	4	2
	End of 3rd Week	9	8	3

The 20 wounds which were taken as control group were treated only by saline dressing. Of these 11 wounds had significant level of bacterial count (>10^5 CFUs /gm of tissue), 7 wounds had insignificant level of bacterial count (≤10^5 CFUs /gm of tissue) and 2 wounds reported as no growth before starting Saline Dressing. (Chart-3)

At the end of three weeks of saline dressing, 9 wounds still had significant bacterial count, 8 wounds had bacterial count in the insignificant level and only 3 wounds reported as no growth. (Chart-4)

TABLE - 16 : COMPARISON OF REDUCTION IN WOUND SIZE BETWEEN STUDY AND CONTROL GROUPS

Group of Wounds	Mean Surface area of wound in sq .cm (% of wound size)			
	0 week	End of 1st week	End of 2nd week	End of 3rd week
Treatment group (n=20)	84.01 (100%)	62.01 (73.8%)	46.71 (55.6%)	34.17 (40.7%)
Control group (n=20)	87.63 (100%)	71.77 (81.9%)	59.98 (68.4)	49.97 (57%)

Before starting Electrical stimulation mean surface area of the wound was 84.01 sq.cm. End of first week the mean wound size was 62.01sq.cm and it was reduced to 73.8% from its initial wound size. Similarly at the end of second week it was 46.71sq.cm (55.6%). Finally at the end of 3 weeks the wound size was 34.17 sq.cm and it was reduced to 40.7% from its initial wound size.

In the control group wounds treated with only saline dressing, mean surface area of the wound was 87.63 sq.cm before treatment. End of first week the mean wound size was 71.77sq.cm and it was reduced to 81.9% from its initial wound size. Similarly at the end of second week it was 59.98 sq.cm (68.4%). Finally at the end of 3 weeks, the wound size was 49.97 sq.cm and it was reduced to 57% from its initial wound size.

TABLE - 17 : HEALING RATE OF STUDY AND CONTROL GROUPS

Group of patients	Healing rate per week	Average healing rate per week
Study group Electrical stimulation	14.9%-26.2%	19.7%
Control group Saline dressing	11.4%-18.1%	14.3%

Clinical observations

Patients who were treated with electrical stimulation had significant decrease in pain, edema and exudates. The healing rates of the wounds treated with electrical stimulation were significantly better when compared to the control. The average healing rate of the wound treated by electrical stimulation was 19.7 % per week, and that of the control was 14.3% per week.

The healing rate of different type of chronic wounds were analyzed and it was found that all types of chronic wounds showed better response with electrical stimulation than control. (chart 5)

TABLE-18 : CORRELATION OF WOUND SIZE AND BACTERIAL BIOBURDEN BEFORE AND AFTER ES TREATMENT

Electrical Stimulation	Surface area of wound in Sq.cm (% of wound size) Mean value	% patients with Significant bacterial count(>10^5 CFUs /gm of tissue)
Before Starting Treatment	84.01 (100%)	60%
After 3 weeks of Treatment	34.17 (40.7%)	5%

Clinical Microbiological correlation of Chronic Wound Healing:

The clinically assessed wound perimetric measurements of study group patients were positively correlated with their semi quantitative bacterial culture assay reports. Before starting treatment the mean surface area of the wound in the study group was 84.01 sq.cm and 60% of these wounds had significant bacterial count. At the end of 3 weeks of electrical stimulation the wound size was reduced to 34.17% and similarly their significant bacterial count was also reduced to 5% .Statistical analysis revealed that the wound healing rate showed a strong inverse relationship with the bacterial count converted to log CFU.(Correlation coefficient r= 0.4017)

TABLE- 19 : SKIN GRAFT SURVIVAL RATE IN RELATION TO BACTERIAL BIOBURDEN

Total No of wounds had Electrical stimulation	Eligible for skin grafting ($\leq 10^5$ CFUs)	100% Graft Survival	Success Rate
20	19	18	94.7%

After 3 weeks of electrical stimulation, 19 out of 20 chronic healing wounds (No growth in 12 wounds; 7 wounds had Insignificant Bacterial Count) eligible for split skin grafting based on the bioburden which denotes in significant bacterial count. ($\leq 10^5$ CFUs)

Among 19 wounds covered with split skin graft, 18 wounds showed 100% Graft Survival with a success rate of 94.7%.

RESULTS OF INVITRO STUDY ON THE ANTIBACTERIAL EFFECTS OF ELECTRICAL STIMULATION:

The electrical stimulation type used in this invitro study was Low voltage pulsed current (LVPC) which delivered biphasic symmetrical rectangular pulses with an amplitude of 40 mA and at a pulse frequency of 128 pulses per second (pps) for 2 hrs duration. The stainless-steel and silver electrodes were used separately with LVPC to explore the antibacterial effects of electrical stimulation on the ATCC strains of Staphylococcus aureus, Pseudomonas aeruginosa and Klebsiella pneumoniae. Growth inhibition of the test organisms represented by the measurements of the zone of inhibition. This inhibition was judged by both the extent of the clear zone and sterility of the region by subculture tests. The results were analyzed as follows.

TABLE - 20 : ANTIBACTERIAL EFFECTS OF ES

Type of Electrode	Zone of Inhibition in mm (Mean value)			
	Pseudomonas aeruginosa	Klebsiella pneumoniae	Staph. aureus	Control
Stainless Steel wire	7	5	3	--
Silver electrode	11	6	4	--

TABLE – 21 : ELECTRO CHEMICAL EFFECTS OF ELECTRODES

Type of Electrode	Chemical and Physical changes Found at Electrodes				
	Pole	Corrosion	Discoloration	Gas bubbles	pH
Stainless Steel wire	±	Small Pitting	Present	Nil	6.2-8.0
Silver electrode	±	Nil	Nil	Nil	6.2-8.0

Results with Stainless-steel Electrodes:

Stainless-steel electrodes were used with LVPC in this initial part of the study and zone of inhibition present predominantly around one electrode since the changing polarity nature of the biphasic pulsed current. The Mean for the diameter of zone of inhibition for Pseudomonas aeruginosa was 7mm; for Klebsiella pnumoniae it was 5 mm; and 3 mm for Staphylococcus aureus. Brownish discoloration of the media and small pitting was present at the electrode which was dominant in all the three Petri dishes inoculated with test

organisms. Subcultures confirmed the sterile nature of the clear zones around the electrodes. There were no changes at the electrodes in the control plates with the organisms which were not electrically stimulated.

Results with Silver Electrodes:

19G silver electrodes were used with LVPC for demonstration of antibacterial effects of the above said organisms using the same methodology. Here also the zone of inhibition present predominantly around one electrode since the changing polarity of the biphasic pulse current, but this dominant electrode effect was not marked in the Pseudomonas culture plate in which the size of zone of inhibition was equal around both electrodes. The Mean for the diameter of zone of inhibition for Pseudomonas aeruginosa was 11mm; for Klebsiella pneumoniae it was 6 mm; and 4 mm for Staphylococcus aureus. Neither media discoloration nor corrosion was observed at both silver electrodes. Subcultures confirmed the sterile nature of the clear zones around the electrodes. The uninoculated MHA plate with the electrodes insitu which was given electrical stimulation subjected at the end of 1 hr for the pH estimation. The pH paper strip was applied around the electrodes immediately after the termination of LVPC and it indicated yellowish green color which denotes a pH range of 6.5 to 7.2. It confirmed that no change in the pH medium after electrical stimulation.

DISCUSSION

Chronic non healing wounds remain one of the most costly unsolved problems in health care today. The management of these non healing wounds involves various departmental interactions and patients full cooperation. Worldwide, diabetic foot and chronic leg ulcers are the major medical, social, and economic problem and are the leading cause of hospitalization for patients with diabetes.

Another important predisposing factor for chronic non healing wounds is the ageing population. WHO report of ageing statistics[92] reveals that the number of people aged over 60 years is expected to reach two billion by 2050.This shows that the incidence of chronic wounds will increase enormously in the near feature.

In the present study indicated that chronic non healing wounds are the most common presentation among diabetic patients. This was correlated with the study by Dormandy[26] et al who pointed out that in Britain, more hospital beds are occupied by diabetics with non healing foot ulcer complications than by those with all other complications of diabetes put together. The prevalence of chronic wounds was more common in the age group between 41-50 yrs in this study.

Rapid slide smear Gram stain technique & Aerobic culture assay:

Out of 160 direct smears by Gram's staining, 77smears showed positive screening in the rapid slide smear technique and 83 were reported as negative smear in this present study.

Among the 160 wound specimens, 77 specimens were both smear and culture positive.47 specimens were smear negative but culture positive.36 specimens were negative for both smear and culture techniques.

This rapid slide technique is reliable in positive detection if the specimen has critical level of bioburden (>10^5 CFUs /gm of tissue). Levine[54] et al demonstrated a microbial load of ≥10^6 organisms per quantitative swab taken from open burn wounds when bacterial cells were observed in a Gram stained smear prepared from the same sample in his study. His observations enhanced the view of slide smear Gram staining technique which was shown to reliably predict the critical colonization (>1x10^5CFUs/ gram of tissue).

Out of 160 wound biopsy tissue specimens collected from both study and control groups, a total of 207 aerobic isolates were obtained. 36 specimens yielded no growth. Among the 207 isolates, 130(62.8%) were gram negative bacilli and 77(37.2%) were gram positive cocci. This was correlated with Arti Kapil[4] et al study in which gram negative bacilli were most frequently isolated (51.4%) followed by gram positive cocci.(33.3%)

Out of the 207 isolates, Staphylococcus aureus 52 (32.5%) was the predominant organism. This correlates with the studies by P.G.Bowler[11]et al (43%); Pathare[69] et al (42.1%); Itzhak Brook (60.4%); and Ananthakrishnan Ramani[2] et al (60%) all these studies isolated Staphylococcus aureus as the predominant organism.

Pseudomonas aeruginosa was another frequently identified organism which was found in 7-33% ulcers in studies by Bowler, P.G & Davies[11], Hansson[33] et al and Schmidt et al. In this present study also Pseudomonas aeruginosa isolates were 36 (22.5%).

Among the enterobacteriaceae 24(15%) were Escherichia coli; 15(9.37%) were Klebsiella pneumoniae; 14(8.75%) were Klebsiella oxytoca; 14(8.75%) were Proteus mirabilis; 12(7.5%) were Proteus vulgaris; 4(2.5%) were Citrobacter freundii; 4(2.5 %) were Morganella morganii; and 3(1.87%) were Citrobacter koseri in the present study. Klebsiella species, Escherichia coli and Proteus spp were the predominant group among enterobacteriaceae in this present study. This correlates with the study by Arti Kapil[4] et al in which the Proteus species and Escherichia coli were common among the gram negative aerobes. It also correlates with the study by Pathare[69] NA who isolated Proteus spp 16.69%, Klebsiella spp 13.97% and Escherichia coli 8.89% from chronic diabetic foot wounds.

In this present study, Enterococcus spp 9(5.62%) and Proteus spp (7.5%) were isolated from diabetic and ischemic ulcers. C.S. Sharp[76] et al who isolated more number of Enterococci and Proteus spp (23.1%) from infected diabetic gangrene.

Poly microbial Prevalence of Isolates:

Among the 124 culture positives from 160 specimens, 83 cultures yielded two isolates and 41 yielded single isolate per specimen. This yielded on average of 1.7 aerobic bacterial species per specimen in this present study. This correlates with the study of Anandi et al [1] who obtained an average of 2 bacterial species/specimen. This is in low incidence to the study of Diane M.Citron[25] (3.8 bacterial species/ specimen), Pathare[69] N.A. study (3 bacterial species /specimen) Ananthakrishnan Ramani et al [4] study (2.97 bacterial species/ specimen), and Arti Kapil et al [4] study (2.3 bacterial species/ specimen). In the present study the specimens were subjected only for aerobic culture techniques since the wounds were superficial open wounds. In the Study by

Anandi[1] et al, no anaerobe was isolated from diabetic ulcers which categorized in to grade 0 and 1. On the other hand patients with cellulites and gangrene had more than 5 types of aerobes mixed with anaerobes in her study.

Polymicrobial etiology was most common in the diabetic ulcers in the present study. This was endorsed by the studies by Wheat[90] et al; Pathare[69] et al and Jonesetal[41] et al.

Pseudomonas aeruginosa, Staphylococcus aureus and Proteus spp were the predominant type which combined with others like Klebsiella spp, Escherichia coli, CONS and Enterococci in this study. This clearly indicated that bacterial synergy plays an important role in the non healing of chronic wounds. The more common presentation of Staphylococcus species and Pseudomonas aeruginosa in the chronic non healing wounds was the ability of these colonizing bacteria to establish and proliferate in a biofilm in the wound.

Antibiotic Sensitivity pattern of Isolates from chronic non healing Wounds:

Out of 52 Staphylococcus aureus isolated predominantly in this study, 33(63.46%) were oxacillin sensitive; and 19 (36.54%) isolates were found to be MRSA. This was correlated with Colsky[19] et al who found as many as half of all S.aureus isolates from hospitalized patients with leg ulcers to be methicillin resistant S.aureus (MRSA). A study by Tentolouris[81] et al found 40% of S.aureus isolated from infected foot ulcers to be MRSA.

All the isolates of Enterococci were 100% sensitive to penicillin, erythromycin and amikacin.

In this present study out of 36 Pseudomonas aeruginosa, 26 (72.22%) were sensitive to amikacin, 19 (52.77%) were sensitive to ciprofloxacin, 15(41.66%) were sensitive Ceftazidime. All these isolates were resistant to cefotaxime and cotrimoxazole. Colsky[19] et al supported this by his study which showed more than one-third of P.aeruginosa isolates to be resistant to ciprofloxacin, a commonly used anti pseudomonad antibody. Tammelin [81]et al found 21.7% of Pseudomonas species were resistant to a clinically relevant antibiotic. All the 4 isolates of Acinetobacter species were 100% sensitive to both ciprofloxacin and amikacin, but all of them were resistant to cefotaxime and ceftazidime.

Out of 90 enterobacteriaceae isolates, 51(56.6%) isolates were sensitive to cefotaxime and ceftazidime. 39(43.4%) enterobacteriaceae isolates were presumptive ESBL producer from the chronic non healing wounds in this present study. In India the prevalence rate of ESBL producing enterobacteriaceae varies in different institutions from 28% to 84% [34] whereas in the US it varies from 0 to 25%

In this present study among the 39 ESBL producers; Proteus vulgaris isolates 8 (66.66%), Escherichia coli isolates 12(50%) Klebsiella pneumoniae isolates 7(46.66%), Klebsiella oxytoca isolates 6(42.85%), Proteus mirabilis isolates 6(42.85%) and Citrobacter freundii isolate 1(25%) detected as ESBL producers. In the study by Ashwins[83] et al Escherichia coli 58.06% and Klebsiella pneumoniae isolates were 43.75% which correlated with the present study. Another study by Varaiya[86] et al coincided with this study by ESBL production was observed in 46.51% of Escherichia coli isolates and 44.44% of Klebsiella pneumoniae isolates.

Importance of Bacterial Bioburden:

In a critically colonized state there will be multiplication of organisms without invasion but interfering with wound healing and patient may not display classical signs of infection. So the critical colonization is currently better explained from a microbiological perspective than from a clinical perspective. The studies done by various authors like RJ.White[91] et al; Stuart Enoch[79] et al; Andrew Kingsley[3]; and Heggers.P. [34] Et al reinforced the importance of estimation of bioburden along with the qualitative microbiology for the betterment of patients with chronic non healing wounds.

In this present study quantitative microbiology was applied not only to assess the initial bioburden but it also helped to compare the effects of Electrical stimulation on the chronic non healing wounds with the control. Previously lot of clinical studies[7, 28, and 31,58,63,94] using various types of electrical stimulation for the management of non healing wounds kept the wound size as the main indicator for assessing the efficacy of electrical stimulation with the control wounds. But only in this present study, the antibacterial effect of electrical stimulation (estimation of bioburden) along with the wound size was used to compare with the control group.

Semi quantitative aerobic bacterial culture assay:

In this present study out of the 20 wounds from study group, the semi quantitative aerobic bacterial culture assay revealed that 12 wounds had significant bacterial count ($>10^5$CFUs /gm of tissue), 5 wounds had insignificant level of bacterial count ($\leq 10^5$CFUs/gm of tissue), and 3 wounds had no growth before starting electrical stimulation treatment. At the end of 1st week after starting electrical stimulation, the number of wounds with

significant count remained unchanged. But at the end of 2nd week, the number of wounds with significant count decreased to half (6 in number). The wounds that had significant bacterial count further reduced at the end of 3 weeks of electrical stimulation, finally only one wound had significant bacterial count ($>10^5$CFUs /gm of tissue).

The 20 wounds which were taken as control group were treated only by saline dressing. Of these 11 wounds had significant level of bacterial count, 7 wounds had insignificant level of bacterial count and 2 wounds reported as no growth before starting saline dressing. The number of wounds with significant count increased to 13 and 14 after starting saline dressing at the end of 1st & 2nd week respectively. The number of wounds with significant count finally stayed at 9 out of 11 in the control group.

Sterility of the wound achieved by the electrical stimulation treatment was also very impressive in that before starting electrical stimulation there were only 3 wounds were reported as no growth but at the end of electrical stimulation treatment, 12 wounds were reported as no growth.This was significant in comparing the control group who had saline dressing alone in which 2 wounds had no growth at the beginning, but even after 3 weeks of saline dressing treatment only one wound was added in the list of no growths. This semi quantitative aerobic bacterial assay clearly indicated that electrical stimulation treatment accelerated the wound healing through their antibacterial effects which lead to the effective reduction in the wound bioburden level in addition to their effects like increased blood flow to the wound; Promotion of phagocytosis; Enhancement of tissue oxygenation; attraction and stimulation of more fibroblast and epithelial cell to the wound site and reduces wound edema perhaps from reduced micro vascular leakage.

Comparison of Mean bacterial count (log CFU) between test and control group was statistically significant.(P=0.0015)

Wound Perimetry Results:

Before starting Electrical stimulation mean surface area of the wound was 84.01 sq.cm. After the electrical stimulation treatment the mean wound size was markedly reduced; end of first week 62.01sq.cm (73.8% from its initial wound size); end of second week it was 46.71sq.cm (55.6%). Finally at the end of 3 weeks the wound size was 34.17 sq.cm and it was reduced to 40.7% from its initial wound size.

Where as in the control wounds with only saline dressing; the reduction in wound size was comparatively less than that of study group. Before saline dressing the wound size was 87.63 sq.cm. End of first week 71.77sq.cm (81.9% from its initial wound size); end of second week 59.98 sq.cm (68.4%). Finally at the end of 3 weeks, the wound size was 49.97 sq.cm and it was reduced to 57% from its initial wound size.

Comparison of Mean reduction in wound size between test and control group was statistically significant ($P < 0.005$)

Excellent reduction in wound size by electrical stimulation treatment in this present study was endorsed by various other clinical studies discussed in (Table 22).

TABLE - 22 : EFFECTS OF ELECTRICAL STIMULATION ON CHRONIC WOUND HEALING IN DIFFERENT PULSED CURRENT CLINICAL STUDIES

Author-Reference	Electrical Stimulation Type	Dosage Duration	Wound Type	Study / Control Groups	Number of wounds	reported outcome
Feedar[28] et al	LVPC-Monophasicathode initially	35 mA @ 128 pps 30 minute BID/ 30 days	Mixed types	LVPC/ control (Sham)	26 24	14% 8.25% Healing Rate/wk
Mulder[63] etal	LVPC Monophasicathode	30,35 or 40 mA @ 128 pps, 30mnts/BID	Mixed types	LVPC/ control (Sham)	26 24	36% 13% Healing Rate/wk
Genizkow et al[31]	LVPC Monophasicathode	Regimen similar to Feedar et al	Decubitus Ulcer	LVPC/ control (Sham)	21 19	12.5% 5.8% Healing Rate/wk
Wood[94] et al	LVPC Monophasicathode	300µA then 600 µA @ 0.8 pps	Decubitus Ulcer	LVPC/ control (Sham)	43 31	73% 13% (Decreas wound size)
Baker[7] et al	LVPC-Biphasic	A:asymmetrica 50pps,100µs pd B: symmetrical 50 pps,300 µs C: microcurrent 1mA,1pps,10 µs	Diabetic ulcers	LVPC A B C Control	29 24 20 19	27% 16% 17% 17% Healing Rate/wk
Lundeberg et al[58]	LVPC-Biphasic	80 pps, 1 ms PD 20 mints/BID	Diabetic ulcers	LVPC Control (Sham)	32 32	12% ES vs. 7% Sham % ulcer healed At 4 wk
Present study	LVPC-Biphasic	40mA, 128 pps Rectangular pulses, 40mnts daily for 3weeks	Mixed types	LVPC/ Control (saline dressing	20 20	14.9% - 26.2% 11.4%- 18.1% Healing Rate/wk

LVPC-Low voltage Pulsed current; pps-pulses per second; Pd-each pulse duration.

The studies by Feedar[28] et al; Mulder[63] and Gentzknow[31] et al used similar treatment protocols of approximately30 mA, 64-128 pps applied 30

minutes twice daily to the wounds for a minimum period of 1 month. They used low voltage monophasic pulsed current. In this present study Low voltage pulsed current (LVPC) was used with biphasic symmetrical rectangular pulses in an amplitude of 40 mA and at a frequency of 128 pulses per second (pps) for 40 minutes duration daily for a period of 3 weeks. All these studies including the present study the healing rates of the wounds treated with electrical stimulation were significantly better when compared to the control. The average healing rate of the wound treated by electrical stimulation was 19.7 % per week, and that of the control was 14.3% per week in the present study. A healing rate of 15% per week is the accepted standard for chronic non-healing wounds. The rate of healing closer to standard level in control in the study can be explained by the fact that all these wounds were superficial.

Clinico Microbiological Correlation:

In this present study effect of electrical stimulation on improving wound healing is judged by two parameters

1. Clinical improvement in the form of decreased surface area of the wound by weekly wound perimetry.

2. Estimation of bacterial bioburden by Semi quantitative aerobic bacterial culture assay weekly.

Before starting treatment the mean surface area of the wound in the study group was 84.01 sq.cm and 60% of these wounds had significant bacterial count. At the end of 3 weeks of electrical stimulation, the wound size was reduced to 37.17% and similarly their significant bacterial count was also reduced to 5%. This result showed a clear positive correlation between the bioburden and the wound size and it implies that reduction in wound bioburden

lead to the reduction in wound size which leads to good healing rate. This was supported by Ling Xu[55] et al; they found that diabetic ulcer healing rate increased with the control of bioburden in their study.

Skin graft Success Rate:

After 3 weeks of electrical stimulation, 19 out of 20 chronic healing wounds (No growth in 12 wounds; 7 wounds had Insignificant Bacterial Count) were eligible for split skin grafting based on the bioburden level of $\leq 10^5$ CFUs In this study among 19 wounds covered with split skin graft, 18 wounds showed 100% Graft Survival with a success rate of 94.7%. This was correlated with Krizek[49] et al study in which those wounds that were grafted when the wound biopsies were determined to be less than or equal to 10^5 CFUs per gram of tissue had a 94% take ; those that had higher counts had less than 20% graft survival.

Effects of Electrical stimulation on Bacterial Viability: An In vitro study

In this present study, exposure to low voltage pulsed current (LVPC) resulted in a marked zone of inhibition around the electrodes in the Mueller Hinton agar plates inoculated with ATCC strains of Staphylococcus aureus, Pseudomonas aeruginosa and ESBL Klebsiella pneumoniae.

With the stainless Steel wires, zone of inhibition of growth (Mean) for Pseudomonas aeruginosa was 7mm; for Klebsielle it was 5mm and for Staphylococcus aureus 3mm and with Silver electrodes the effects were 11mm of zone of inhibition for Pseudomonas aeruginosa, 6 mm for Klebsiella and 4mm for Staphylococcus aureus. Small zone of inhibition for Staphylococcus aureus may be due to their thick cell wall nature, compared to the response shown by gram negative bacilli for the electrical stimulation. ESBL producing

K.pneumoniae also shown a zone of inhibition which indicate the antibacterial effects of electrical stimulation even on the drug resistant organisms.

TABLE-23 : IN VITRO STUDIES ON THE ANTIBACTERIAL EFFECTS OF ES

Author Reference	Pathogen	Current	Stimulation Parameters	Polarity/ Effect	Electrode Type	Antibacterial Effects
Rowley[75]	*Escher. coli*	DC AC	mA1.0,14, 140 1, 10,30, 60 pps mA = 15 or 30	Cathode None	Platinum Platinum	bacteriostatic No bacteriostatic effect
Baranco et al[8]	*S.aureus* Esch.coli P.aerugin P.vulgaris	DC	µA = 40 and 400	Anode (negligible gas and pH Δ)	Silver, platinum, gold, stainless steel	bacteriostatic
Wai-Kin Liu[89] et al	S.aureus S.epidermi	DC	10-100 µA	Cathode	Carbon catheters	bactericidal
Kincaid and Lavoie[45]	S.aureus Esch.coli P.aerugin	HVPC	150,200, 250,300 V 120 PPS	Cathode	stainless steel	bacteriostatic
Szuminsky et al[80]	S.aureus Klebsiella Esch.coli P.aerugin	HVPC	500 V 120 pps	Anode Cathode (gas and pH Δ both poles	stainless steel stainless steel	All inhibited At both poles
Laika D. Roy[51]	Esch.coli S.epidermi	LVPC	6 mA, 20 mA	Anode Cathode	stainless steel	bactericidal
Georg Daeschlein et al[32]	3 GNBs 3 GPCs	LVPC	Derma pulse system	Anode Cathode	Not mentioned	bactericidal
Present study	S.aureus Klebsiell P.aerugin	LVPC	40 mA 128 pps 2 hours	Changing Polarity	stainless steel Silver	bactericidal

In this study, the effects of LVPC with silver electrodes were more pronounced than with stainless steel electrode and the adverse electrochemical effects like media discoloration, corrosion were present around stainless steel electrodes only.

These results were well correlated with the previous study done by S.D.Barranco et al. He compared 5 electrode types which were used with weak

direct current on 4 bacterial species. He observed that silver electrode was extremely bacteriostatic even at the lowest current when used as the anode. He found that antibacterial effects were mainly due to the electrochemically injected silver from the anode.

Biofilm formation by chronic wound colonizers like Pseudomonas aeruginosa, Staphylococcus aureus and S.epidermidis posing a big challenge since these biofilm bacteria are resistant to the antimicrobials which are in turn needed in higher concentrations to kill them. The studies by Costerton et al and Del Pozo et al documented the electrical enhancement of efficacy of antibiotics in killing biofilm bacteria. This has been termed as “bioelectric effect’.

In a recent study by Del Pozo et al demonstrated the “electricidal effect’ on the reduction of Staphylococcus and Pseudomonas biofilms by prolonged exposure to low –intensity electrical current without any antibiotics in a sophisticated in vitro model in which biofilms were grown on Teflon coupons in a semi synthetic medium.

This present study can also be extended further in this field of biofilm bacteria’s and electrical stimulation.

SUMMARY

A total of 160 tissue biopsy specimens were collected from 38 patients with 40 chronic non healing superficial wounds admitted to department of Plastic surgery and the specimens were processed and subjected for qualitative and semi quantitative aerobic bacterial culture assays at the department of microbiology, Stanley medical college, Chennai from June 2008 to May 2009.

Among the 40 wounds, 20 wounds were treated with electrical stimulation for 3 weeks duration and kept as study group and another 20 wounds were treated with saline dressing alone and they were kept as control group. None of these groups were treated with either antibiotics or topical antiseptics.

Diabetic ulcers were the commonest presentation and most of the patients were in the age group between 41- 50 years and majority of them were males.

Out of 207 aerobic isolalates, Staphylococcus aureus was the predominant isolate, followed by Pseudomonas aeruginosa, Klebsiella spp, Proteus spp, Escherichia coli etc.

Out of 124 positive cultures, 83 were polymicrobial and 41were monomicrobial in nature.

The prevalence of MRSA in this hospitalized patients with chronic non healing wound were 36.54%.

Among the enterobacteriaceae 43.4% were found to be ESBL producers by preliminary screening, disk diffusion method.

All the isolates of Pseudomonas aeruginosa were resistant to cefotaxime, 72.2% were sensitive to amikacin, 41.7% were sensitive to ceftazidime and 52.7% were sensitive to ciprofloxacin.

Slide Smear technique for rapid assessment of bacterial bioburden showed 77 wound biopsy specimens had significant bacterial count ($>10^5$ CFUs /gm of tissue)

Semi quantitative aerobic bacterial culture assay was done before and after electrical stimulation treatment for the study group revealed that 12 wounds had significant bacterial count ($>10^5$CFUs /gm of tissue), 5 wounds had insignificant level of bacterial count ($\leq 10^5$CFUs/gm of tissue), and 3 wounds had no growth before starting electrical stimulation treatment.

At the end of 3 weeks of electrical stimulation, the bacterial count has decreased and only 1 wound had significant bacterial count, 7 wounds had insignificant level of bacterial count and 12 wounds were reported as no growth.

In the control group before starting Saline Dressing 11 wounds had significant level of bacterial count, 7 wounds had insignificant level of bacterial count, and 2 wound specimens were reported as no growth.

At the end of three weeks of saline dressing, 9 wounds still had significant bacterial count, 8 wounds had bacterial count in the insignificant level and only 3 wounds reported as no growth.

The average healing rate of the wounds treated by electrical stimulation was better with 19.7% per week than that of control wounds which was 14.3% per week.

The reduction of bioburden after electrical stimulation was positively correlated with the reduction in the wound size and showed a strong inverse relationship with wound healing rate.

Out of 20 wounds treated with electrical stimulation, 19 wounds were covered with split skin graft. Among the 19 wounds, 18 wounds showed 100% graft Survival with a success rate of 94.7%.

Effects of electrical stimulation on bacterial viability was demonstrated with Pseudomonas aeruginosa, ESBL Klebsiella pneumoniae and Staphylococcus aureus in a in vitro model which showed a clear zone of inhibition of growth of test organisms around the electrodes in the MHA plates. Silver electrodes showed greater zone of inhibition compared to stainless steel electrodes.

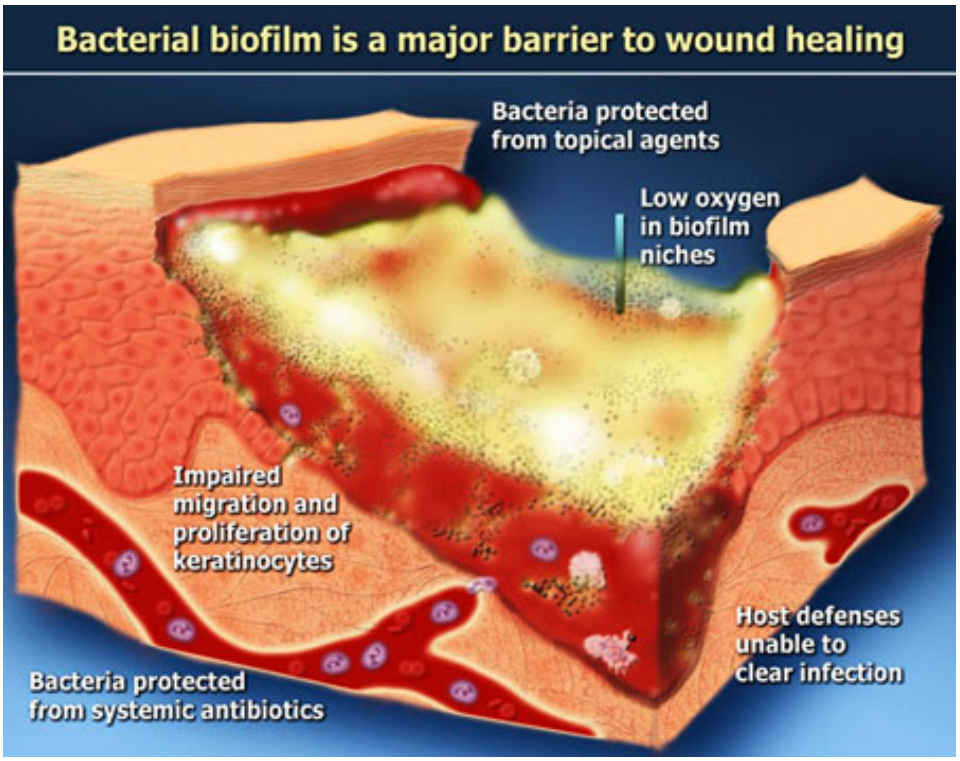
Both the in vivo and in vitro studies clearly documented that the effects of electrical stimulation which lead onto reduction in the bacterial bioburden within the chronic wound tissue will assist in the conversion of chronic inflammation to a resolving inflammatory response which lead on to accelerated wound healing.

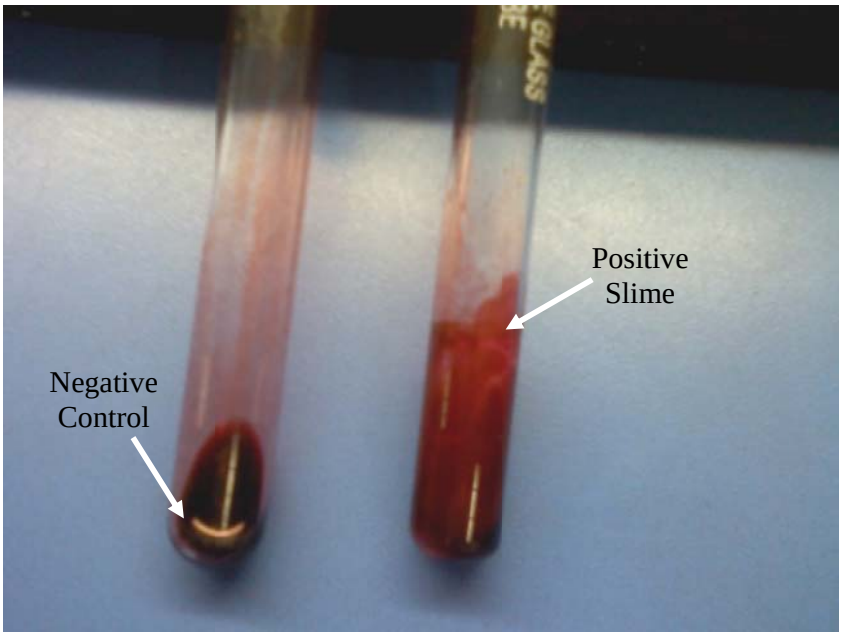
CONCLUSION

- The Microbiological profile of the chronic non healing wounds reveals a critical level of bioburden, Biofilm formation the polymicrobial flora, synergistic relationship between microorganisms and the presence of specific pathogens and their toxins all these causes a state of non healing in the chronic wounds .
- Importance of quantitative microbiology is well reinforced in that it is not only helps to estimate the bioburden but also useful in the quantitative assessment of various treatment modalities out come. This also gives a clue to the clinician in deciding the timing of wound closure because the critical bacterial load of $>10^5$ CFUs /gm of tissue will badly affect the skin graft survival.
- In this era of increasing multi drug resistance & hospital acquired infections; chronic non healing wound patients are clearly a high risk group for acquisition, carriage and dissemination of antibiotic resistant organisms to which antibiotic therapy should not be given merely on clinical grounds alone but it must be validated by the qualitative and quantitative microbiological assays.
- The electricidal effects of electrical stimulation on the bacterial viability are clearly documented by in vivo & in vitro studies.
- The electrical stimulation can be used adjunctively with other standard wound care management to enhance the wound healing.
- This study can also be extended further to assess the effects of electrical stimulation on “Bacterial Biofilms - the major culprits in the Device-related human infections’ which begins with the adhesion of the micro organisms to the biomaterial surface and then this biofilms protect bacteria from host defense mechanisms and conventional antimicrobial agents.

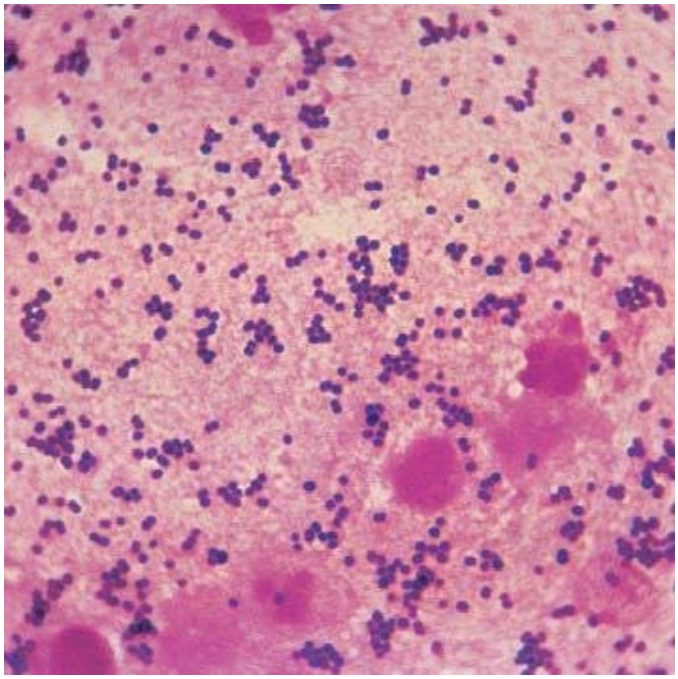
Electrical Stimulation --- Chronic superficial wounds																							
		Semiquantitative assay results				Qualitative assay with Antibiogram													Wound sizeSq.cm				
S.No	Name	Age/Sex	Diag	0 Week	1 Week	2 Week	3 Week	Isolate/s	P/A	E	Co	Ox	Van	Gm	Ak	Cip	CE	CZ	Imi	0 Week	1 week	2 Week	3 Week
1	Daisy	35/F	DU	$6.8x10^4$	$4.9x10^4$	NG	NG	S.aureus	S	S	R	S		S	S	S	S			143	109	78.75	61.5
								E.coli	R		R			S	S	R	R	R	S				
2	Dhanalakshmi	49/F	Au	$8.4x10^6$	$7.5x10^5$	$8.6x10^4$	NG	S.aureus	R	R	R	R	S	R	R	S	R			70	54.5	30.5	20
3	Piyare John	76/M	Vu	$2x10^6$	$1.5x10^6$	$6.8x10^5$	$2.4x10^4$	P.vulga	R		R			R	R	R	R	R	S	18.5	13.2	10.8	6.6
								E.coli	R		R			R	S	S	R	R	S				
4	Saravanan	47/M	Au	$9.8x10^5$	$1.5x10^6$	$1.8x10^6$	$6.4x10^6$	E.coli	R		R			R	S	S	S	S		74	58.75	51.25	35.35
								CONS	R	R	S	R	S	S	S	R	R						
5	Kanniyan	70/M	DU	$1.8x10^6$	$1.2x10^5$	$2.7x10^4$	NG	k.pneum	R		R			R	S	S	R	R	S	89	67.5	53.25	39.25
								P.aerug			R			S	S	S	R	R					
6	Meganathan	51/M	Cu + L	$2x10^6$	$8.5x10^5$	$8x10^5$	$7.4x10^4$	S.aureus	R	R	S	S		R	S	S	S			126.25	93.5	71.33	55.5
7	Padma	55/F	Du	$3x10^6$	$6.5x10^5$	$3.1x10^4$	$2.4x10^4$	S.aureus	R	S	R	S		S	S	S	S			116	86.3	64.3	48.75
								k.pneum	R		R			R	S	S	S	S					
8	Muniammal	70/F	Cu + L	$2x10^6$	$5x10^5$	NG	NG	P.mirab	R		R			R	R	S	R	R	S	65	41.25	32	19
								k.oxyto	R		R			R	R	S	R	R	S				
9	Samsudeen	52/M	U.Scar	$1.5x10^6$	$8.9x10^5$	NG	NG	P.aerug			R			R	S	S	R	R		38	24	18.25	15
								S.aureus	S	S	S	S		S	S	S	S						
10	Thirumoorhty	28/M	Au	$4.2x10^4$	$3.8x10^4$	NG	NG	P.aerug	R		R			R	S	R	R	S		138.75	105.3	78.5	56.5
11	Vijayan	43/M	Au	$5x10^4$	$2.7x10^4$	NG	NG	E.coli	R		R			R	S	R	R	R	S	78	56.75	43.5	31.75
								S.aureus	R	S	R	S		R	R	S	S						
12	Palani	28/M	Vu	NG	NG	NG	NG	NG												86.25	66.25	49.25	36.25
13	Padmanabhan	65/M	Du	$6x10^6$	$1.9x10^6$	$1.2x10^5$	$3.7x10^4$	P.mirab	R		R			R	S	R	S	S		75	51.9	37.75	28.75
								CONS	R	R	R	R	S	R	S	S	S						
14	Ravi	47/M	DU	$2x10^6$	$5x10^5$	$2.2x10^5$	$1.8x10^4$	k.pneum	R		R			R	S	R	S	S		86.25	68.25	51.25	36.25
								P.mirab	R		R			R	S	R	S	S					
15	Sasi	36/M	Vu	NG	NG	NG	NG	NG												137	99.3	76.5	55.75

		Semiquantitative assay results				Qualitative assay with Antibiogram													Wound sizeSq.cm				
Sl.No.	Name	Age/Sex	Diag	0 Week	1 Week	2 Week	3 Week	Isolate/s	P/A	E	Co	Ox	Van	Gm	Ak	Cip	CE	CZ	Imi	0 Week			
16	Senthil	26/M	Du	$2.1x10^4$	$1.8x10^4$	NG	NG	P.aerug	R		R			R	R	S	R	R		48.75	38.15	29.75	20.75
								Enteroc	S	S	R				S								
17	Vasu	47/M	Cu + L	$3x10^6$	$2x10^6$	$4.1x10^5$	$1.5x10^4$	P.aerug			R			R	R	R	R	S		67	47.5	34.25	25.5
								Acineto			R			R	S	S	R	R					
18	Rosammal	61/F	Du	NG	NG	NG	NG	NG												34.5	22.25	15.75	11.25
19	Suresh	23/M	Vu	$1.2x10^6$	$7x10^5$	$5x10^5$	$2.6x10^4$	S.aureus	R	R	R	S		S	S	S	S			126	94.25	73.5	55.5
								P.aerug	R		R			R	S	R	R						
20	Annamalai	51/M	Au	$3.3x10^4$	$1.6x10^4$	NG	NG	S.aureus	R	R	R	S		S	S	R	S			63	42.5	33.25	25.25
								P.aerug	R		R			R	S	R	R	S					


Bacterial biofilm is a major barrier to wound healing
Bacteria protected from topical agents
Low oxygen in biofilm niches
Impaired migration and proliferation of keratinocytes
Host defenses unable to clear infection
Bacteria protected from systemic antibiotics



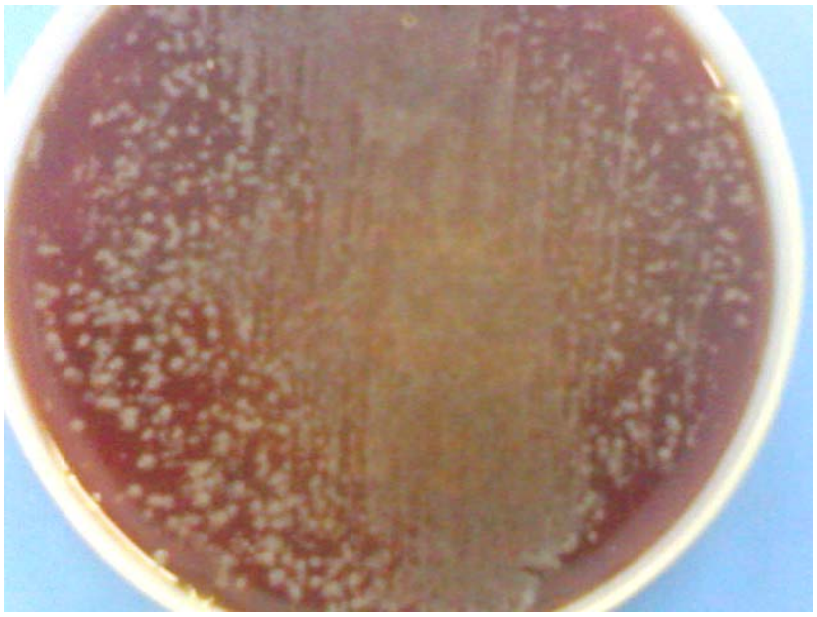
INVITRO TUBE METHOD OF SLIME PRODUCTION IN *P.aeruginosa* FROM WOUND BIOPSY



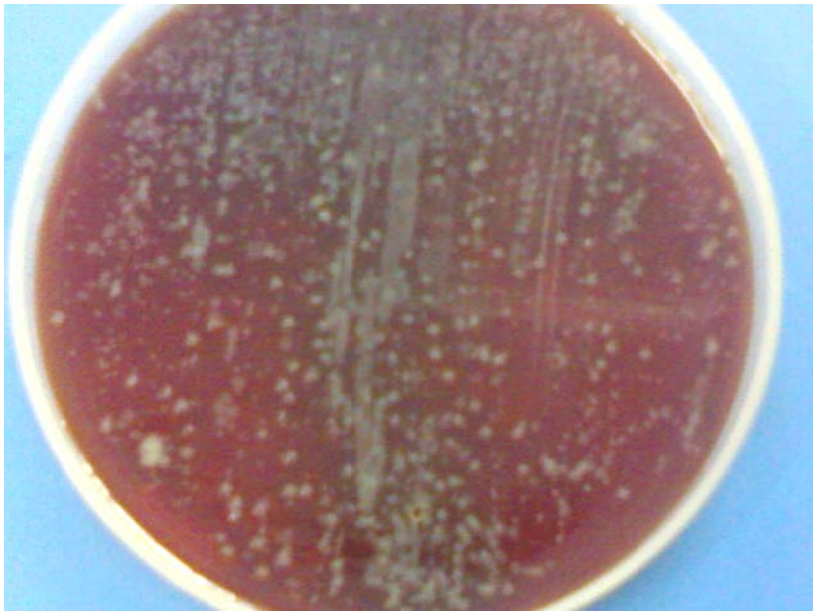
DIRECT GRAMS STAINING SHOWING GRAM POSITIVE COCCI IN CLUSTERS

STAPHYLOCOCCUS AUREUS ON BLOOD AGAR

SEMIQUANTITATIVE BACTERIAL CULTURE ASSAY



0.1ml Inoculum



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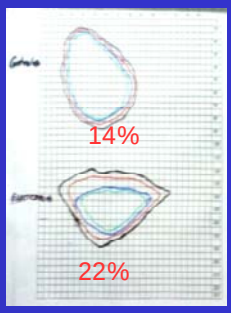
ELECTRICAL VS CONTROL HEALING RATE

DAILY SALINE DRESSING

ELECTRICAL STIMULATION



DAY 1 1 WEEK 3 WEEKS

ELECTRICAL VS CONTROL HEALING RATE

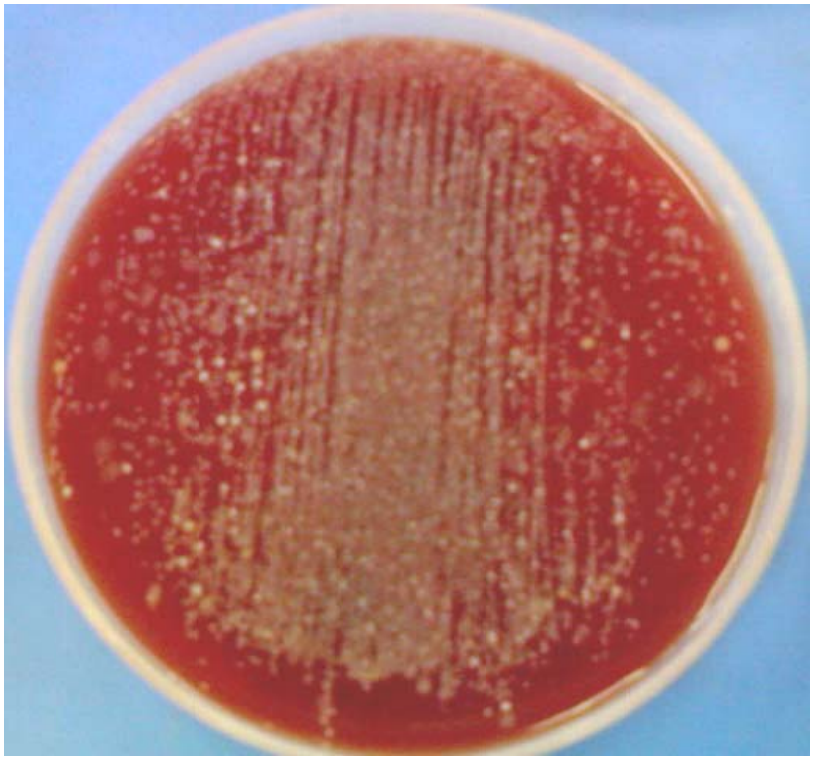
CONTROL – DAY 1

CONTROL – 1 WEEK

CONTROL – 3 WEEKS

ELECT – DAY 1 ELECT – 1 WEEK ELECT – 3 WEEKS ELECT – POST OP

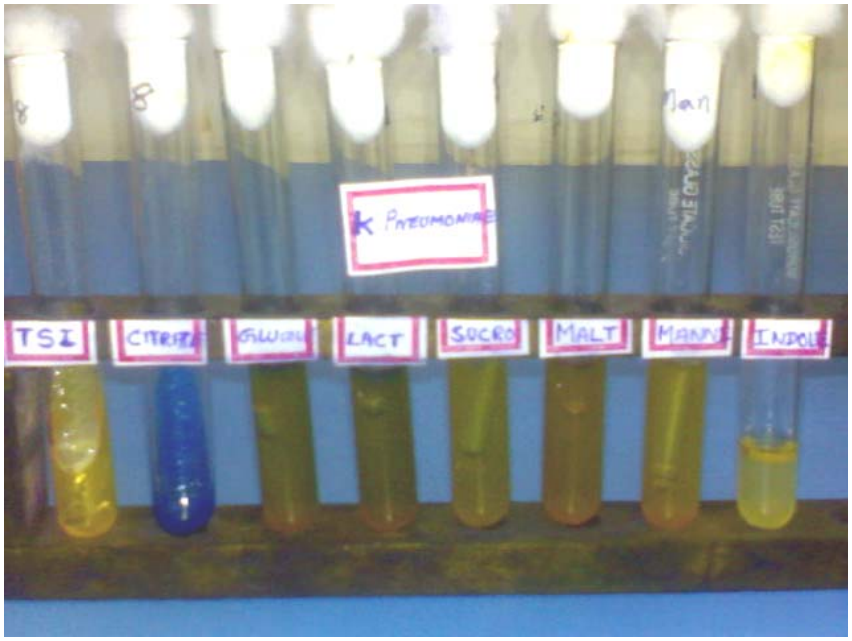
SEMIQUANTITATIVE BACTERIAL CULTURE ASSAY



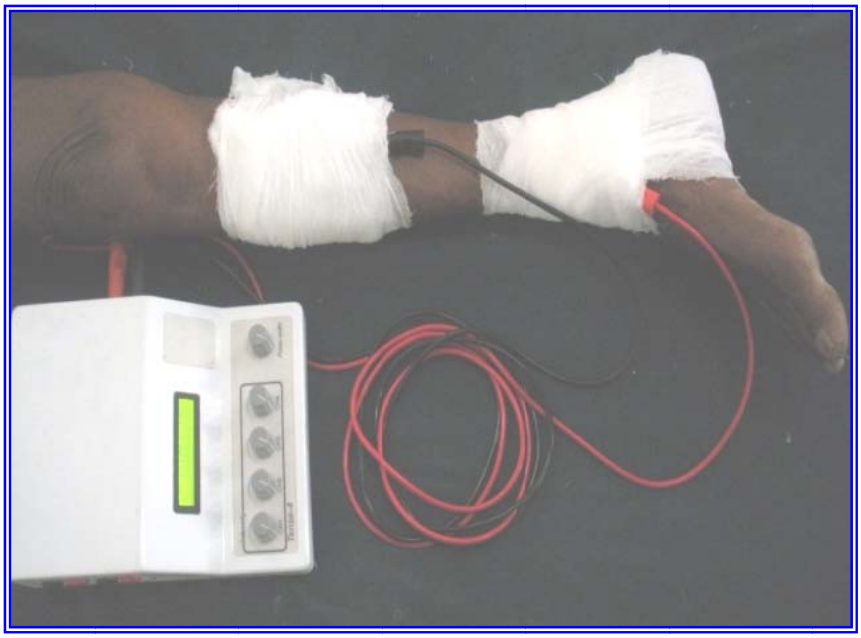
BEFORE ELECTRICAL STIMULATION 0.1ml Inoculum

AFTER 1 WEEK OF ELECTRICAL STIMULATION 0.1ml Inoculum

KLEBSIELLA PNEUMONIAE ON Mac CONKEY AGAR

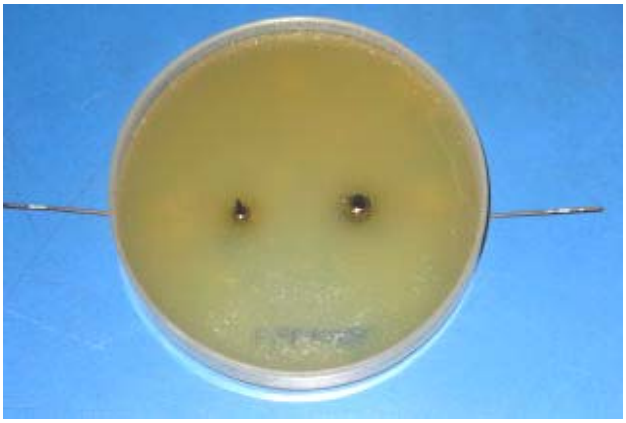


BIOCHEMICAL PROPERTIES OF *K.Pneumoniae*

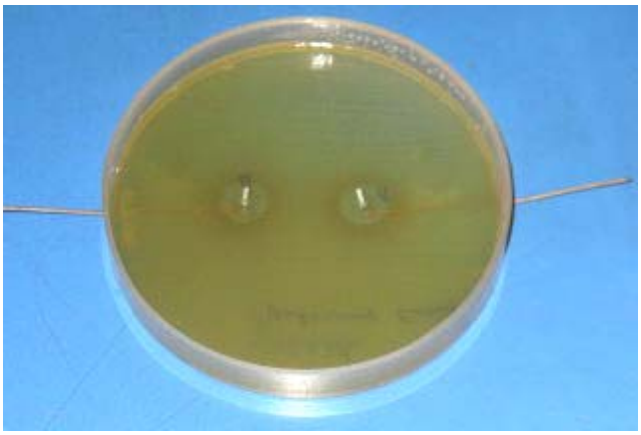


CLINICAL SETTINGS FOR ELECTRICAL STIMULATION

INVITRO SETUP FOR ANTIBACTERIAL EFFECTS OF ELECTRICAL STIMULATION

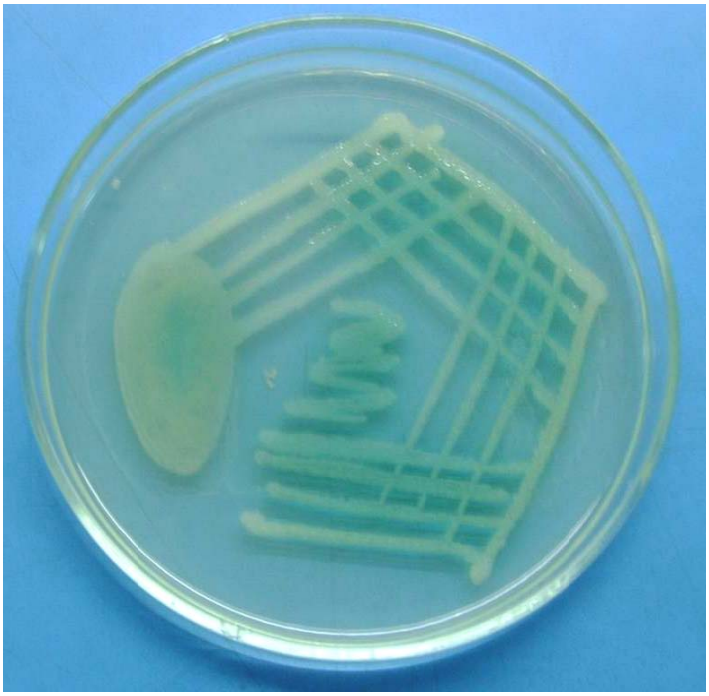


ELECTRODE CORROSION OF STAINLESS STEEL WIRE ON *K.Pneumoniae*

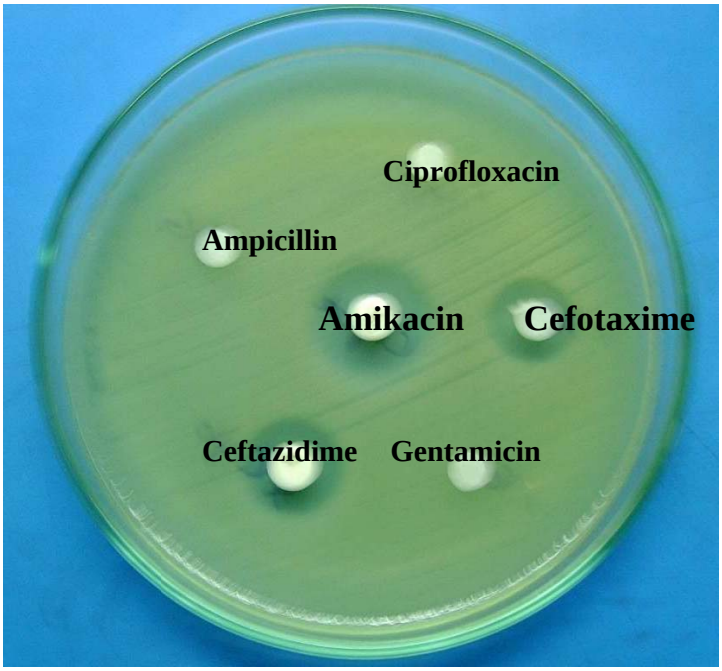


ZONE OF INHIBITION OF *P.aeruginosa* GROWTH AFTER ELECTRICAL STIMULATION WITH SILVER ELECTROD

ZONE OF INHIBITION OF *S.aureus* GROWTH AFTER ELECTRICAL STIMULATION WITH SILVER ELECTRODE

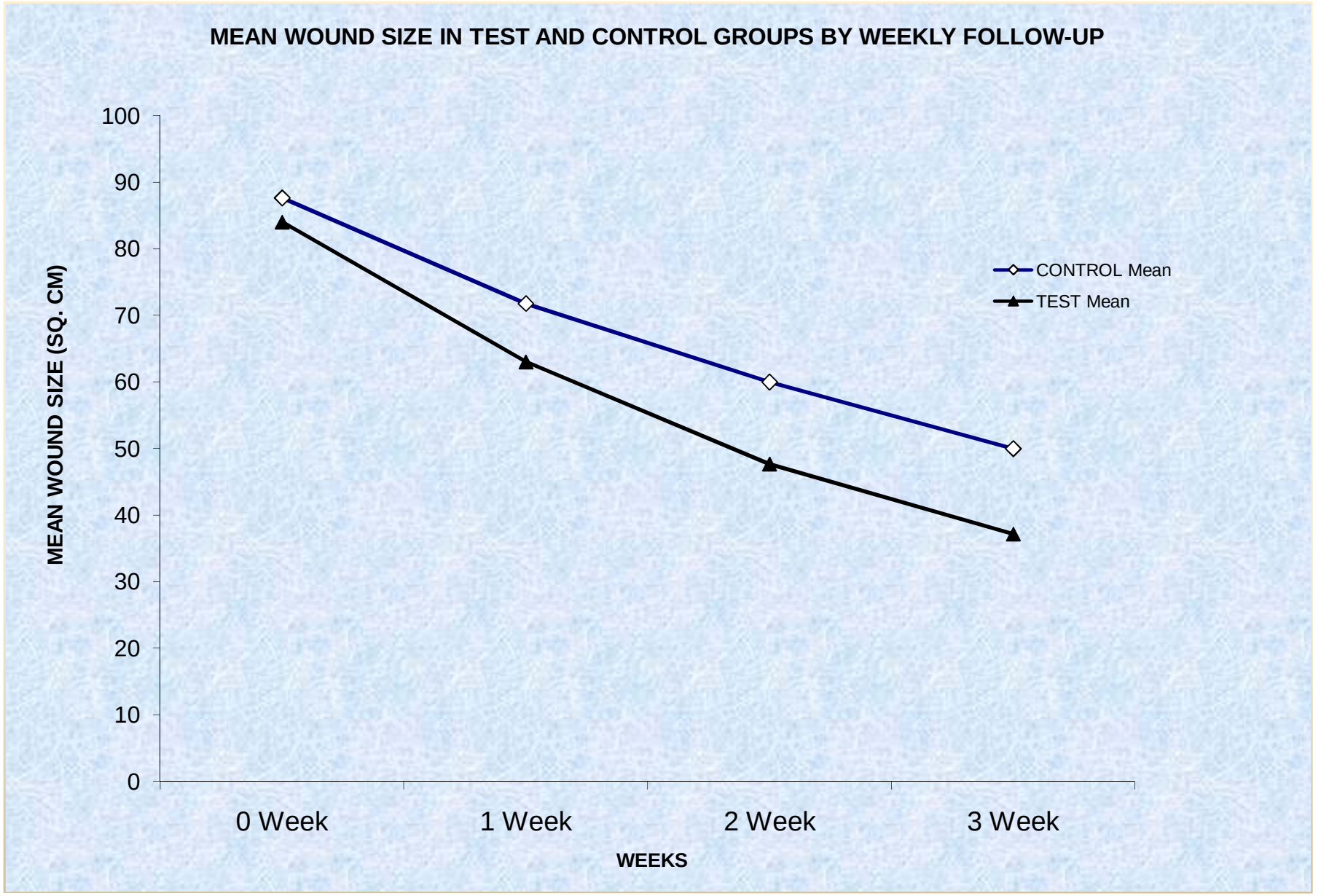


PSEUDOMONAS AERUGINOSA ON NUTRIENT AGAR



Orginsm was resistant to Cefotaxime and Ceftazidime but was sensitive to Amikacin

ANTIBIOGRAM SHOWING RESISTANCE PATTERN


MEAN WOUND SIZE IN TEST AND CONTROL GROUPS BY WEEKLY FOLLOW-UP
MEAN WOUND SIZE (SQ. CM)
100
90
80
70
60
50
40
30
20
10
0
0 Week
1 Week
2 Week
3 Week
WEEKS
CONTROL Mean
TEST Mean

COMPARISON OF HEALING RATES OF DIFFERENT TYPES OF CHRONIC WOUNDS

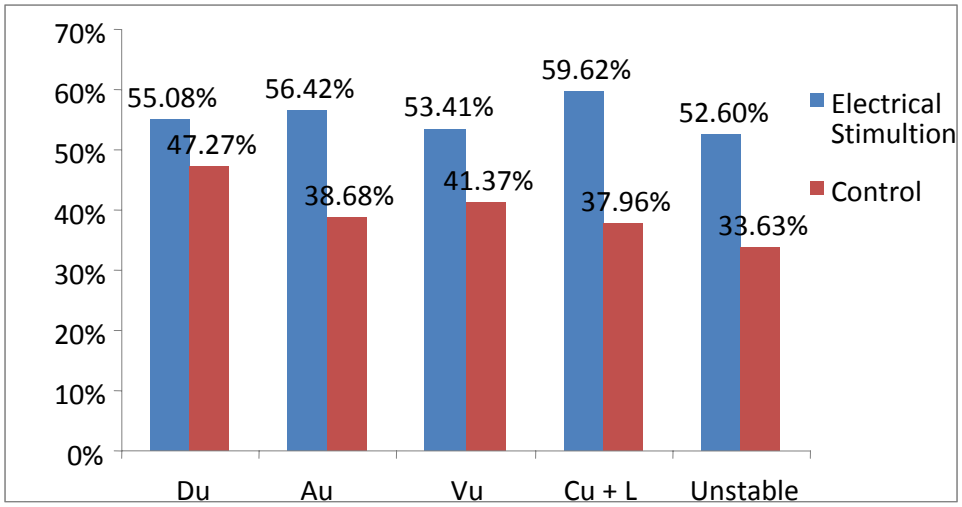


CHART - 5

Du - Diabetic ulcer

Au - Arterial ulcer

Vu - Venous ulcer

Cu+L - Chronic ulcer with lymph edema

Unstable - Unstable scar

CHART- 1

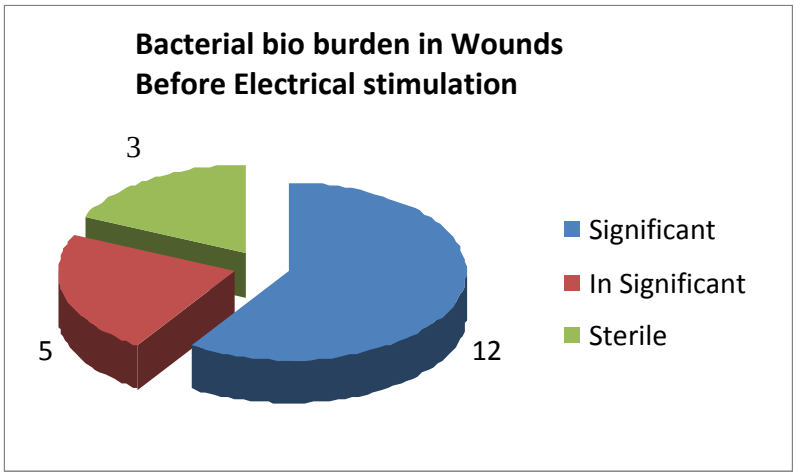


CHART - 2

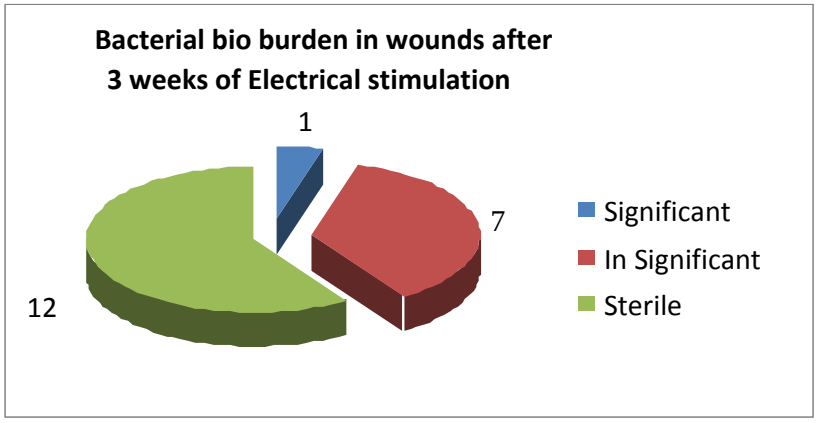


CHART- 3

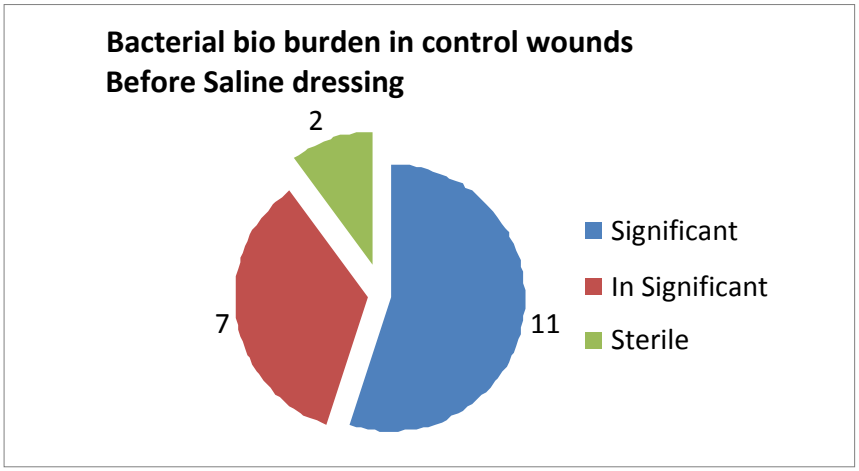


CHART - 4

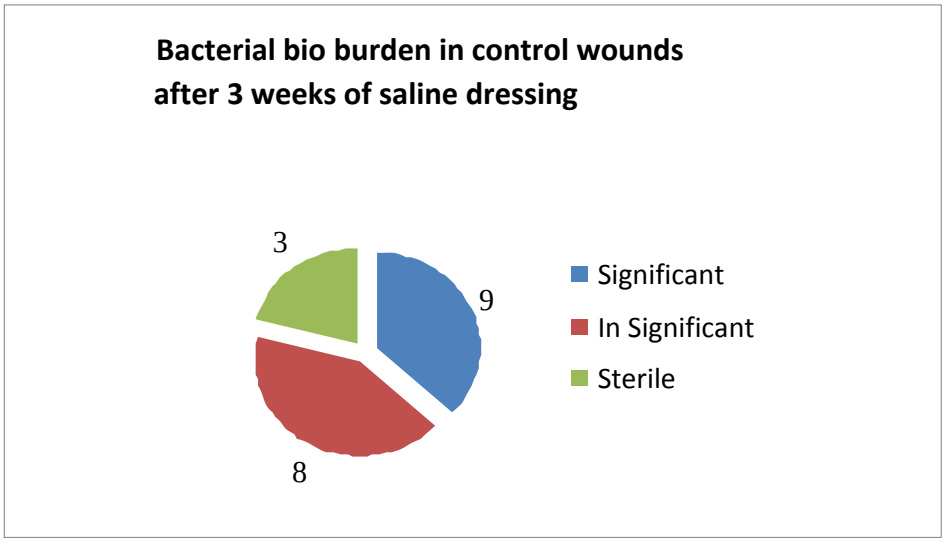
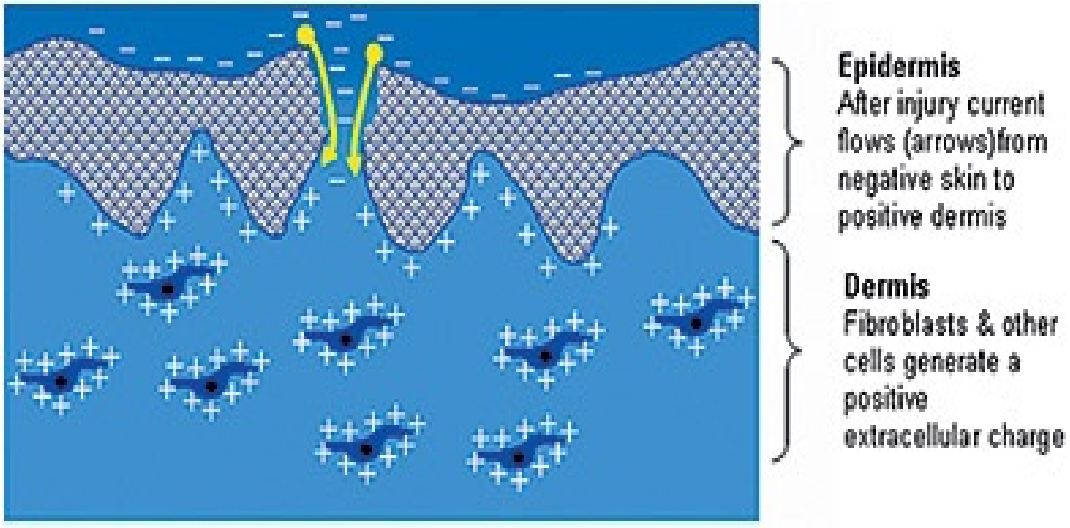


FIGURE 1: THE CURRENT OF INJURY.



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KEY TO MASTER CHART

CU + L	-	Chronic ulcer with Lymphedema
Du	-	Diabetic Ulcer
VU	-	Venous Ulcer
AU	-	Arterial Ulcer
U.Scar	-	Unstable Scar
P	-	Penicillin
A	-	Ampicillin
E	-	Erythromycin
Co	-	Cotrimaxazole
Ox	-	Oxacillin
Van	-	Vancomycin
Gm	-	Gentamycin
Ak	-	Amikacin
Cip	-	Ciprofloxacin
CE	-	Cephotaxime
CZ	-	Ceftazidime
Imi	-	Imipenem
NG	-	No growth
R	-	Resistant
S	-	Sensitive